UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Evaluation of Cytotoxic and Genotoxic Effects of Microcystin-LR in *Saccharomyces cerevisiae*

Sara Estrela Soares Barreiros

Mestrado em Biologia Humana e Ambiente

Dissertação orientada por:
Doutora Elisabete Valério
Professora Doutora Deodália Dias

2017

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Evaluation of Cytotoxic and Genotoxic Effects of Microcystin-LR in *Saccharomyces cerevisiae*

Mestrado em Biologia Humana e Ambiente

Sara Estrela Soares Barreiros

Thesis dissertation oriented per:

Doutora Elisabete Valério
Departamento de Saúde Ambiental – Instituto Nacional de Saúde Dr. Ricardo Jorge

Professora Doutora Deodália Dias
Departamento de Biologia Animal – Faculdade de Ciências – Universidade de Lisboa

2017

Previous notes

This project had the support of the co-supervisor Doutora Maria João Silva (Departamento de Genética, Instituto Nacional de Saúde Doutor Ricardo Jorge).

The thesis dissertation references and bibliography were written in accordance with *Toxicon* Journal criteria.

This work led to the following communication:

Panel communication in international conference:

Barreiros, S., Silva, M.J., Valério, E. 2016. Evaluating cytotoxic and genotoxic effects of microcystin using *Saccharomyces cerevisiæ* as eukaryotic cell model. 3rd International Conference on Occupational and Environmental Toxicology (ICOETox 2016), Porto, Portugal, June 21-23 2016 (ICOETox; Abstract book, Page 75).

Acknowledgments

I would first like to thank my thesis advisor Doutora Elisabete Valério of the Environmental Health Department at Instituto Nacional de Saúde Doutor Ricardo Jorge. She consistently allowed this paper to be my own work, but steered me in the right the direction whenever she thought I needed it.

I would also like to thank Sérgio Paulino, Doutora Elsa Dias and Mestre Carina Menezes. Their office doors were always open whenever I ran into a trouble spot or had a question about my research or writing.

Secondly I would like to thank the experts who were involved in the validation of some of the project techniques: Doutora Maria João Silva and Doutora Henriqueta Louro of the Human Genetics Department at Instituto Nacional de Saúde Doutor Ricardo Jorge. Even though we could not reach our ambitious goal, without their passionate participation and input, the validation survey would most certainly be a completely failure.

I would like to acknowledge Profª Deodália Dias from Faculdade de Ciências da Universidade de Lisboa for the constant support and sympathy. I am gratefully indebted for her very valuable input on this thesis.

I would like to give my deepest appreciation to my partners in crime, my stress relievers and my lunch companions André, Inês, Andreia, Joana, Catarina and Sara. I will cherish your friendship and never forget all the things you thought me.

Finally, I must express my very profound gratitude to João Carvalho and my family, specially my parents, for providing me with unfailing support, love and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without you. Thank you and I love you with all my heart.

Sumário

Neste trabalho pretendia-se caracterizar os efeitos genotóxicos e citotóxicos de uma toxina (Microcistina-LR) que é produzida por cianobactérias, utilizando como organismo eucariota modelo a levedura *Saccharomyces cerevisiae*.

As cianobactérias são bactérias fotossintéticas existentes frequentemente em ambientes aquáticos, inclusive em água doce. Podem sobreviver em locais com níveis de pH e amplitudes térmicas distintas. Atualmente, são usadas para diversos propósitos, incluindo produção de comida animal, fertilizantes, entre outras. Quando as condições ambientais são propícias (p.e. concentração de nutrientes, temperatura, pH…), as cianobactérias podem crescer massivamente, um fenómeno conhecido por *blooms*, que pode estar associado à produção de toxinas por parte das cianobactérias, prejudiciais a diversas espécies, incluindo o ser humano, tornando-se uma questão de preocupação ambiental e de saúde pública.

As microcistinas (MC) são hepatotoxinas, o que significa que o órgão alvo principal de atuação é o fígado, produzidas por cianobactérias. A sua fórmula química é $C_{49}H_{74}N_{10}O_{12}$, tendo no entanto dois resíduos variáveis, que são responsáveis pelas cerca de 80 variedades da toxina. A MC utilizada neste trabalho tem um resíduo de Leucina e Arginina, sendo assim designada por MC-LR. De entre as MCs, a microcistina-LR (MC-LR), produzida por várias espécies, em particular pela espécie *Microcystis aeruginosa*, é a mais abundante, a mais tóxica e também a que se encontra mais bem estudada. Estas foram algumas das razões pelas quais esta toxina foi escolhida. As MCs são péptidos cíclicos que têm uma elevada afinidade para as fosfatases proteicas de Serina/Treonina (PPs), nomeadamente a PP1 e PP2A, agindo assim como inibidores das mesmas especialmente da última. É a partir destas interações que ocorrem uma série de eventos responsáveis pelos efeitos citotóxicos e genotóxicos das MC em células animais. Para além de afetar o fígado, sabe-se que as MC induzem o aumento de espécies reativas de oxigénio (ROS), no entanto ainda não se conseguiu uma caracterização completa dos efeitos destas toxinas.

A exposição Humana é um risco atual sendo que as pessoas podem ter contacto com a toxina, especialmente quando praticam desportos aquáticos, como nadar ou praticar surf em águas balneares contaminada, ou ingerir águas contaminadas que não foram devidamente tratadas para consumo humano. Se houver contacto com elevadas doses da toxina, podem ocorrem sintomas como náuseas, vómitos e danos no fígado, no entanto se o contacto com MCs for crónico e em doses baixas não ocorrem sintomas físicos, mas há danos cumulativos no fígado ao longo do tempo. Devido a estas características a Organização Mundial de Saúde (OMS) estabeleceu um valor máximo para a toxina em águas para consumo, sendo este de 1 µg/L, que se encontra também na Legislação Nacional aplicável.

Desde que a OMS estabeleceu um valor máximo para microcistinas em águas para consumo, o contacto Humano com concentrações elevadas desta toxina tem sido raro. No entanto, a exposição prolongada a doses baixas pode vir a ser problemática, pois é assintomática.

Este projeto pretendeu esclarecer alguns dos mecanismos moleculares de toxicidade da MC-LR em células animais usando *Saccharomyces cerevisiae* como organismo eucariota modelo. Tentou-se caracterizar os efeitos genotóxicos e citotóxicos da exposição a doses relevantes do ponto de vista ambiental de MC-LR em *S. cerevisiae*.

Para avaliar os efeitos citotóxicos da MC-LR, foi utilizado um ensaio de viabilidade celular que determina a capacidade funcional das mitocôndrias, o ensaio de MTT, após exposição das leveduras a diferentes concentrações de MC-LR durante 4 horas. Como controlo positivo utilizou-se o SDS (Dodecil Sulfato de Sódio) e H_2O_2 (Peróxido de Hidrogénio) onde se observou uma relação significativa na dose-resposta, sendo que quanto maior a percentagem de SDS e H_2O_2 usada, maior foi o decréscimo de viabilidade celular. Quando se utilizaram diferentes concentrações de MC-LR observou-se um aumento da absorvância quanto maior fosse a concentração usada, um efeito inverso ao esperado.

Por outro lado, tentou-se ainda adaptar o ensaio de eletroforese de célula única em gel (ensaio do cometa), convencionalmente realizado em células de mamífero, às células de *Saccharomyces cerevisiae*, a fim de quantificar quebras induzidas no DNA. Começou-se por adotar o protocolo de Oliveira et al. (2012) e expor as células a diferentes concentrações do controlo positivo, H_2O_2. Durante a otimização do processo, alterou-se principalmente a composição dos vários componentes do ensaio, o tampão de eletroforese, a solução de lise, a concentração do gel, a duração da eletroforese e a voltagem da mesma. Em alguns ensaios conseguiu-se expor o DNA das células, confirmando que a liticase funcionava pois houve digestão da parede celular, mas não houve migração do mesmo, o que significa que o protocolo ainda não está totalmente otimizado.

Os efeitos genotóxicos foram avaliados através da análise da alteração dos níveis de expressão dos genes *Rad27*, *Apn1*, *Apn2*, *Ntg1* e *Ntg2* do sistema de reparação de DNA de *S.cerevisiae*, BER e do gene *Cdc55*, que codifica a proteína fosfatase PP2A, através da técnica de Real-Time PCR. Utilizaram-se os genes *Alg9* e *Taf10* como referência, para normalizar os níveis de expressão. *S. cerevisiae* foi exposta a quatro concentrações de MC-LR; 1 nM, 10 nM, 100 nM e 1 µM, e os resultados obtidos foram comparados com a situação controlo (sem toxina). O gene *Apn1* mostrou uma tendência para ser subexpresso quando as células foram expostas a concentrações mais baixas de MC-LR e sobrexpresso para concentrações mais elevadas. O gene *Apn2* mostrou uma tendência para ser subexpresso em qualquer uma das concentrações de MC-LR testadas. O gene *Rad27* mostrou uma relação de dose-resposta onde existe uma tendência para quanto maior for a concentração de MC-LR maior é a expressão do gene. O gene *Ntg1* tem tendência a ser subexpesso em todas as concentrações de MC-LR. O gene *Ntg2* mostra uma relação dose-resposta clara, onde quanto maior for a concentração de microcistina menor é a expressão génica. O gene *Cdc55* sofre uma repressão génica quando exposto a qualquer concentração de microcistina. Apesar dos resultados obtidos serem para já tendências, demonstram ser interessantes e estão relativamente de acordo com o espectável.

Como conclusões finais a retirar deste trabalho, pelos resultados obtidos com o MTT, aparentemente a microcistina não parece ser citotóxica para *S.cerevisiae*, contudo apesar de se ter verificado que o MTT é um método que funciona muito bem em *S. cerevisiae*, poderá não ter sido o método mais adequado para analisar a toxicidade de MC-LR, pelo que é necessária uma confirmação dos resultados obtidos através de outras técnicas que avaliam a viabilidade celular. Em relação ao ensaio Cometa, os resultados não foram conclusivos, possivelmente devido à dificuldade em otimizar o método para leveduras, nomeadamente na migração do DNA no campo elétrico. No entanto, o método é bastante intuitivo e seria bastante interessante que futuramente viesse a ser otimizado para leveduras. Verificou-se que *S. cerevisiae* aparenta ser suscetível a MC-LR, confirmado pelos efeitos genotóxicos. Em relação ao método de RT-qPCR conseguiu-se obter tendências na expressão génica, quando comparadas com a situação controlo. Este método permitiu ver que a microcistina parece afetar ambas as vias do sistema de reparação de DNA BER, mas de forma diferente. No entanto é necessário realizar mais ensaios com

maior número de réplicas biológicas para que se possa proceder ao tratamento estatístico e obter resultados mais consistentes.

Apesar da dificuldade em reproduzir alguns métodos em leveduras, tudo indica que a microcistina-LR pode desempenhar um papel crítico na toxicidade de células eucariotas. Este trabalho permitiu contribuir um pouco mais um campo de estudo ainda pouco conhecido, utilizando *S.cerevisiae* como organismo modelo eucariota.

Palavras-chave: *Saccharomyces cerevisiae*, microcistina, genotoxicidade, citotoxicidade

Abstract

Microcystins (MC) are hepatotoxins produced by cyanobacteria. Among the MCs, the microcystin-LR (MC-LR), produced by several cyanobacterial species, especially by the species *Microcystis aeruginosa,* is the most abundant and also the most well studied cyanotoxin. MCs are cyclic peptides which have high affinity for protein phosphatases Serine/Threonine (PPs), namely PP1 and PP2A, thus acting as their inhibitors, especially of the last one. It is from these interactions that a series of events occur which are responsible for the MCs cytotoxic and genotoxic effects on animal cells. It is also known that MCs induce oxidative stress in cells due to the production of reactive oxygen species (ROS), however a complete characterization of the effects of these toxins has not yet been obtained.

This project intends to clarify some of the molecular mechanisms of MC-LR toxicity in animal cells using *Saccharomyces cerevisiae* as an eukaryotic organism model.

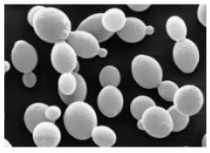
To evaluate the cytotoxic effects of MC-LR, a cell viability assay was used to determine the functional capacity of the mitochondria, the MTT assay, after exposing the yeasts to different concentrations of MC-LR for 4 hours. Genotoxic effects were evaluated by gene expression studies for genes *Rad27*, *Apn1*, *Apn2*, *Ntg1* and *Ntg2* (from the BER DNA repair system) and *Cdc55* gene which encodes the PP2A phosphatase protein, using the Real-Time qPCR technique. The reference genes used for expression normalization were *Alg9* and *Taf10*. Furthermore, it was attempted to adapt the single cell gel electrophoresis assay (comet assay), conventionally performed on mammalian cells, to *Saccharomyces cerevisiae* cells, in order to quantify induced DNA breaks.

MTT was optimized and successfully used in *S. cerevisiae.* Apparently, MC-LR is not cytotoxic for *Saccharomyces cerevisiae,* although these results should be confirmed with other methods that accessed cell viability. Regarding the Comet assay, the results were not conclusive, possibly due to the difficulty in optimizing the method when applied to yeast cells, particularly in the DNA migration on the electric field. However, the first two steps of the YCA protocol were optimized. Concerning the RT-qPCR method it was possible to obtain tendencies in the gene expression levels, when compared with the control situation, thus revealing that MC-LR affects differently both BER pathways.

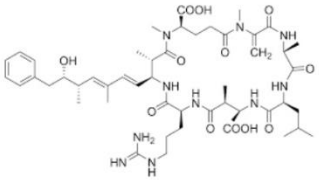
Despite the difficulty of reproducing some methods in yeast cells, it appears that microcystin-LR plays a critical role in the toxicity of eukaryotic cells. This work allowed us to contribute with a little more information to a still relative unknown study field.

Keywords: *Saccharomyces cerevisiae*, microcystin, genotoxicity, cytotoxicity

Graphical Abstract



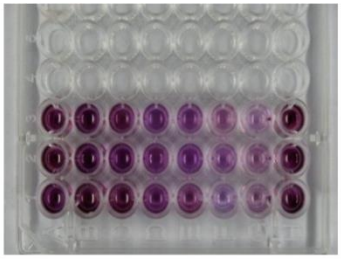
Saccharomyces cerevisiae



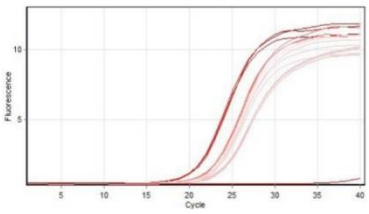
Microcystin-LR

Cytotoxic effects

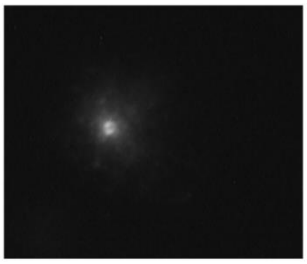
Genotoxic effects



MTT assay

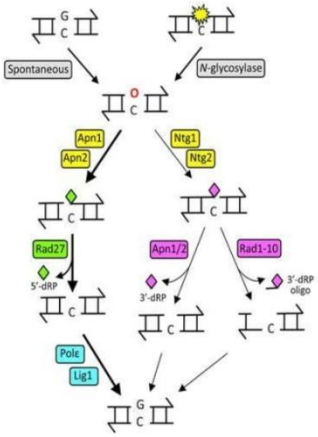


RT-qPCR



Comet assay

Target Genes:
APN1
APN2
Rad27
Ntg1
Ntg2



BER system

\+ Target gene:
cdc55

Table of contents

Figures Index

Tables Index

Abbreviations and Acronyms

AP – apyrimidinic/apurinic

BER – Base Excision Repair

BLAST - Basic Local Alignment Search Tool

Bp – Base pair

CA – Comet assay

cDNA - complementary DNA

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DNase − Desoxirribonuclease

dNTPs - Nucleoside Triphosphates deoxyribose

EDTA – Ethylenediamine tetraacetic acid

GSH – Glutathione

H_2O_2 – Hydrogen Peroxide

LDH – Lactate Dehydrogenase

Mbp – Mega base pairs (=1,000,000 base pairs)

MC - Microcystin

MC-LR – Microcystin – LR (Leucine-Arginine)

mRNA - messenger RNA

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH - Nicotinamide Adenine Dinucleotide

NER – Nucleotide Excision Repair

OATP – Organic Anion Transporting Polypeptides

OD – Optical density

PPs – Protein Phosphatases

RNA – Ribonucleic acid

ROS – Reactive Oxygen Species

RT-qPCR – Real Time quantitative Polymer Chain Reaction

SDS – Sodium Dodecyl Sulfate

Tris-HCl – Tris hydrochloride

WHO – World Health Organization

YCA – Yeast Comet assay

YPD – Yeast Peptone Dextrose

1. Introduction

1.1 Cyanobacteria

Cyanobacteria are photosynthetic bacteria that are part of normal microbial communities, especially in marine and freshwaters, they are responsible for nitrogen, carbon and oxygen dynamics in many aquatic environments (Briand et al., 2003; Zhou et al., 2015). Cyanobacteria can be found in places with different thermal amplitudes such as frozen lakes, to deserts and hot spring mats that can reach 85ºC, although their optimum growth is usually over 15ºC (Zhou et al., 2015). They can survive in acidic bogs, basic, salted and freshwater environments, however they prefer alkaline conditions. Besides living as free-living organisms, they can also form symbiotic associations (Vicente, 2009).

Nowadays, cyanobacteria are used by humans for countless purposes such as food sources for animals, fertilizers and production of health products (Vicente, 2009). More recently, cyanobacteria have become popular study target due to their industrial potential to produce biofuel (Sarmaa et al., 2016).

Some species of cyanobacteria under certain conditions, particularly when high levels of nutrients are available and the water temperature is higher, may proliferate and reach high densities, a phenomenon called blooms (Briand et al., 2003; Zhou et al., 2015).

Cyanobacteria have a great public heath interest since some genera have the ability to produce toxins that may affect aquatic organisms, as well as terrestrial species that come in contact with, or ingest them through the affected waters, such as wildlife, livestock, pets and even humans (Briand et al., 2003). The toxin production by cyanobacteria species is influenced by genetic and environmental factors, including nutrient concentrations, temperature and pH. If the required conditions are not gather the cyanobacteria will not produce toxins (Van der Merwe, 2014).

Diverse incidences of toxic cyanobacterial blooms are increasing in most regions worldwide, causing a growing concern in the community. The so far described episodes of human poisoning have occurred through the contact with contaminated water, including drinking water, recreational water use, contaminated food or dietary supplements and contaminated hemodialysis water (Paerl et al., 2001; Hudnell, 2010).

1.1.1 Occurrence of microcystins

Cyanobacteria can produce different types of toxins (cyanotoxins), that are commonly divided according to the main target organ.

Microcystins (MC) are one of the kind of hepatotoxins, produced by cyanobacteria of the genera *Planktothrix*, *Microcystis*, *Anabaenopsis*, *Nostoc*, *Hapalosiphon*, *Nodularia* and *Anabaena* (Valério et al., 2016). Microcystins are cyclic heptapeptides and the most important cyanotoxins in terms of health impact and water quality, since they are the most frequent and widespread (WHO, 2003). *Microcystis aeruginosa* is one of the most common producer of microcystins (Van der Merwe, 2014). Microcystins general chemical formula is $C_{49}H_{74}N_{10}O_{12,}$ however it has two variable residues, which are responsible for the 80 variants of the toxin (Zhou et al., 2015). The microcystin (MC) used in this study has a leucine and an arginine as residues, MC-LR (Figure 1.1). This variant has been chosen because is the most toxic and one of the most well studied (Campos & Vasconcelos, 2010).

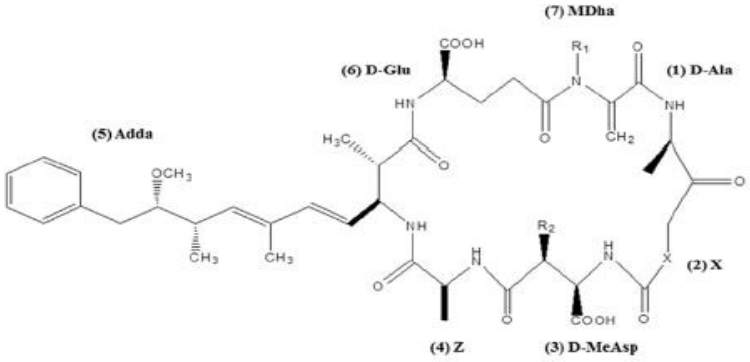


Figure 1.1: *General structure of MCS. For MC-LR the X is Leucine and Z is Arginine (Zhou et al., 2015)*

Microcystins concentrations are generally correlated with cyanobacteria cell density (Van der Merwe, 2014), the greater the number of cyanobacteria, the higher toxin production. Cyanobacteria often develop in water treatment plants, water, which will be used for human consumption. If there is surface water eutrophication at the exit of the treatment plant, cyanobacteria numbers should be accessed. If the number of potentially cyanobacteria producers of microcystins is greater than 2000 cells/ml, sampling frequency should increase and the water should not be considered risk free (Decreto-Lei n.º 164/306, 27 de Agosto, 2007).

Human exposure is an actual risk since people are often in contact with the toxin while doing water sports such as swimming or skiing, in contaminated waters. Other common routes of human exposure include drinking water, contaminated foods or nutritional supplements (Azevedo et al., 2002). One of the most tragic and media case occurrence was in 1996, where 56 patients died in a dialysis center in Brazil. Latter it was confirmed that the dialysis water, from a nearby reservoir with a *Microcystis* bloom, was not fully treated, filtered, and chlorinated. The presence of microcystin was detected in serum and liver tissue of case patients (WHO, 2003; Chen et al., 2009).

Terrestrial animals, including pets, livestock, and wildlife, are typically exposed when drinking from contaminated lakes and ponds. Aquatic species living in contaminated waters may also be affected. The increasing number of artificial new lakes and ponds created for water management, recreation and drinking water for farm animals creates opportunities for microcystin occurrence and exposure in susceptible populations. Potentially poisonous concentrations of microcystin are reached relatively frequently in lakes and ponds with high nutrient concentrations (eutrophic), and it appears to be increasing in most regions due to the expansion of intensive agriculture, industrial development and urbanization (Van der Merwe, 2014).

Microcystin can have catastrophic consequences on human and animal health. If living beings have contact with high doses of microcystin for a short time, physical symptoms appear such as vomit, bleeding, nausea, hallucinations and liver damage, this is considered an acute exposure (Brinkman & Bourne, 2013; WHO, 2003). However, if there is chronic exposure to low doses, there are no immediate physical symptoms but cumulative liver damaged happens. Moreover, MC-LR not only can cause acute poisoning but it has also been associated with a potential to promote cancer in humans, when exposed to repetitive low concentration in drinking water (Chen et al., 2009; Zhou et al., 2015). Therefore, the World Health Organization (WHO) established a guideline value of MC-LR in drinking water, 1 µg/L (WHO, 2003).

1.1.2 Microcystin-LR mechanisms of action

MCs are large molecules, hence, incapable of crossing cell membranes via passive diffusion. Therefore, they require active transport, via specific transporters, through specific organic anion transporting polypeptides (OATPs) (Fisher et al., 2010). The main OATPs congeners used by microcystins are OATP1B1 and OATP1B3, which in humans are located in the liver membrane and kidney cells. That explains the critical effects of this toxin, MC-LR, in the liver (Fisher et al., 2010).

1.1.2.1 MC-LR inhibits protein phosphatases (PPs)

Protein phosphatases (PPs) are found in all tissues and across several species and play a critical role in cells protein activity. They are responsible for protein phosphorylation and dephosphorylation, by removal of their phosphate group. Any disturbance in phosphatases activity will affect cellular homeostasis (internal cell stability) (Zhou et al., 2015; Maynes et al., 2006).

Previous studies in human liver cell lines show different decreases in PP2A activity when treated with different MC-LR concentrations (Sun et al., 2014). They have a high affinity for the protein phosphatases of Serine / Threonine (PPs) family, namely PP1 / PP2A, acting as inhibitors. These interactions are responsible for the cytotoxic and genotoxic effects of MC in animal cells.

Since PP2A is a main target for MC-LR pathway, it was decided to study cdc55 gene which is related to the protein phosphatase and encodes one of the regulatory subunits for PP2A (Valério et al., 2016a). The mammalian homolog is the gene B55 (CDC55, 2017 from http://www.yeastgenome.org/locus/S000003158/overview, accessed on January 2017).

1.1.2.2 MC-LR induces oxidative stress

One of the toxic effects of MC-LR is the induction of an increase of reactive oxygen species (ROS) and glutathione (GSH) depletion. Almost all organisms have anti-oxidant defense systems but continues exposures to MC-LR may disrupt it and lead to the depletion of GSH (Zhou et al., 2015).

Although the mechanism involved in over-production of ROS still remains to be fully undercover, in a preview study Ding and Ong (2003) proposed that MC-LR increased the oxidative stress by two primary pathways; first MC-LR provokes a depletion of GSH which leads to oxidative damage and cell death; secondly it increases the production of ROS by disrupting the electron transport in the mitochondria which leads to the release of apoptotic factors, resulting also in apoptosis (Ding and Nam Ong, 2003). Oxidative stress can cause cell death by apoptosis or necrosis and is related with the mitochondria metabolism (Campos et al., 2010; Valério et al., 2016).

1.2 *Saccharomyces cerevisiae* as a model

Saccharomyces cerevisiae is a yeast from the genera *Saccharomyces* and it is considered a model organism due to its characteristics such as, having a low maintenance, its duplication time is very fast, between 1.25 to 2 hours at 30ºC. Replicative lifespan is about 26 cell divisions (Warringer et al., 2011). Another advantage is having the genome already sequenced and at least 31% of the proteins encoded in the yeast genome have a human orthologue and nearly 50% of human disease genes exhibit yeast orthologues (Menacho-Márquez & Murguía, 2007).

S. cerevisiae has two major DNA repair systems: NER (Nucleotide Excision Repair) and BER (Base Excision Repair). The NER pathway has the ability to remove structures that interfere with base pairing and transcription (Cadet et al., 2005; Friedberg et al., 2006). The BER pathway removes the majority of damaged nucleotides from oxidative DNA lesions, to deamination (Boiteux et al., 2013).

1.2.1 BER DNA repair system

DNA damages are alterations of the chemical structure of DNA, such as a break in the DNA strand, a missing DNA base, or a chemically changed base. DNA damages can occur naturally from metabolic cell activity or induced by several environmental factors such as toxins or radiation. These alterations can change gene function or gene expression if located in coding regions (Bernstein et al., 2013).

The DNA repair system allows the removal of incorrect and damaged nucleotides, preventing potential replication errors and fixation of genetic mutations. Defects in these metabolic processes have, in many cases, been shown to lead to cellular inviability or chromosome instability (Sun et al., 2003).

The BER pathway occurs in five sequential steps requiring the sequential action of several enzymes: (1) a DNA N-glycosylase that releases the damaged base from the deoxyribose, (2) an endonuclease/lyase that nicks the DNA backbone at the resulting apyrimidinic/apurinic (AP) site, (3) a 3’- or 5’-phosphodiesterase that removes the remaining deoxyribose phosphate residue, (4) a DNA polymerase that fills the gap thus created, and (5) a DNA ligase to seal the remaining nick (Hoeijmakers, 2001). The BER process is represented in Figure 1.2, where the AP sites “O” are generated by base loss or by DNA N-glycosylase. The initiation of the repair pathways starts when *Apn1* and *Apn2* nick the backbone on the 5’ side of an AP site, resulting in 5’-dRP which is removed by the *Rad27* 5’-flap endonuclease. The repair pathway can also be initiated by the *Ntg1* or *Ntg2* lyase, which nicks on the 3’ side of lesion. It results in 3’-dRP which can be removed by the 3’-diesterase activity of *Apn1*/*Apn2* or as part of a Rad1-Rad10 generated oligonucleotide. The gap is then filled by a DNA Polymerase, and the backbone is sealed by DNA ligase 1.

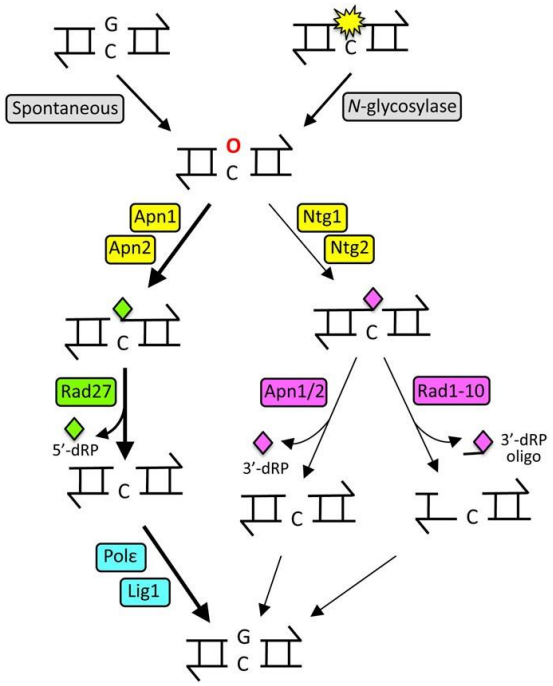


Figure 1.2*: BER pathway (Boiteux et al.,2013)*

Apn1 has the major AP endonuclease activity in *S. cerevisiae* BER system, and has a functional homolog in mammalian - Ape1 (Morris et al., 2012). Although *Apn2* has little influence regarding AP endonuclease activity *in vivo*, it is a highly conserved component of the BER system. Apn2 is better known for playing an important role in oxidative damage repair (Fraser et al., 2003). *Rad27* belongs to the Rad2 nuclease family, and has a human homolog the Fen-1 nuclease. *Rad27* is responsible for regulating the expansion of simple repeat elements and prevent larger duplications. Some studies also show that *Rad27* null mutations are conditionally lethal, which suggest a complementary nuclease activity (Sun et al., 2003). The major properties of BER genes are summarized in Table 1.1 (Boiteux et al., 2013).

Some of these genes (g.e. Fen-1 – mammalian counterpart of gene *Rad27*) play a role in the protection against the development of human diseases that result from repeat instability, such as cancers and Huntington's disease. Which means that changes in the expression levels of these genes (caused by microcystin for example) could be very problematic (Sun et al., 2003).

Table 1.1: *DNA N-glycosylases, AP endonucleases, and end-processing enzymes (Boiteux et al.,2013)*

Gene name	Protein size (kDa)	Properties	Mammalian counterpart
Ntg1	45.5	Bifunctional DNA N-glycosylase/AP lyase. Excision of oxidatively damaged pyrimidines and AP sites in dsDNA	*Nth1*
Ntg2	43.8		

Apn1	41.4	AP endonuclease and 39-phosphodiesterase. Incision of regular and oxidized AP sites. Excision of 39-blocked ends	*Ape1*
Apn2	59.4		*Ape2*
Rad27	43.3	59-Flap endonuclease. Excision of 59- drp after cleavage of AP sites by Apn1 or Apn2	*Fen1*

Legend: dsDNA - double-strand DNA

1.3 Evaluation of cytotoxic effects

Cytotoxicity occurs when a compound is toxic to cells. Cells exposed to these compounds can react in various ways, they may lose membrane integrity and suffer cell lysis; they may stop growing and dividing; or they may suffer apoptosis.

There are numerous ways to measure cytotoxicity, but the most common involve assessment of cell membrane integrity. Membrane integrity can be evaluated using vital dyes (such as propidium iodide) or measuring intracellular enzymes activity (such as Lactate Dehydrogenase (LDH) assay). Other cytotoxicity assays rely on the measurement of cell viability through the incorporation of dyes (e.g. Neutral red assay) or measuring the metabolic activity (e.g. MTT assay).

In this study, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was chosen to access cell viability (Molecular Devices, 2017 from https://www.moleculardevices.com/applications/areas-research/cytotoxicity, accessed on February 2017). MTT assay is a safe, sensitive, *in vitro* assay to measure cell viability or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. In metabolically active cells MTT is reduced by dehydrogenases and reducing agents to insoluble violet-blue formazan dye crystals (Figure 1.3). The lipid soluble formazan product is solubilized with DMSO (Dimethyl sulfoxide), an organic solvent and the color intensity is measured in the spectrophotometer. The absorbance was read at 570nm with a reference wavelength of 690 nm in a micro titer plate reader. The optical density of the control cells were fixed to be 100% viability and the viability percentage of the cells in the other treatment groups were calculated. There is a positive association between the level of formazan formed and cells viability (Van Meerloo et al., 2011).

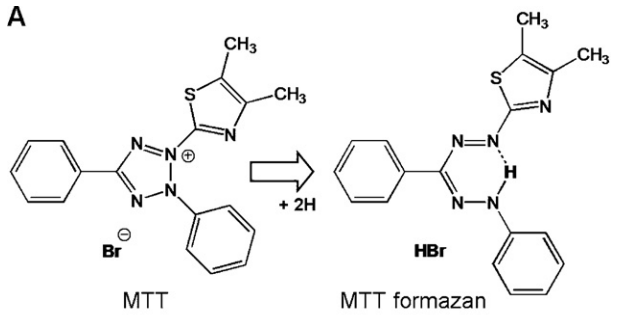


Figure 1.3: *Chemical structure of MTT and the reduced formazan (Stockert et al., 2012)*

Some authors have suggested that the MTT in living cells is reduced by mitochondrial dehydrogenases activities which is not exact since the mitochondria does not show reducing power but oxidizing power. This assumption is still wildly used since mitochondria are depleted of the nicotinamide adenine dinucleotide (NADH) coenzyme, that is the main component reduced in presence of MTT, giving rise to a modification of their staining properties (Stockert et al., 2012). However due to some biochemical evidences other authors assert that the MTT is mainly reduced in the cytoplasm by NADH and from some dehydrogenases associated to the endoplasmic reticulum, endosomes and plasma membrane (Bernas and Dobrucki, 2002). Oxidative stress generates a rapid decrease of mitochondrial NADPH that is followed by a slow recovery, however under more extreme oxidative conditions it may result in cell damage or death (Petrat et al., 2003).

1.4 Evaluation of genotoxic effects

In this study, the genotoxic effects of MC-LR were evaluated by selected gene expression measurement using Real-Time PCR and by the Comet assay.

1.4.1 Comet assay

The single cell gel electrophoresis or Comet assay under alkaline conditions is a simple, rapid and sensitive assay that allows the detection of DNA lesions, including DNA single and double-strand breaks and alkali-labile sites in individual cells (Azevedo et al., 2011; Oliveira et al., 2012; Azqueta et al., 2013).

The Comet assay is based on the differential electrophoretic mobility of DNA across an agarose gel, according to its molecular weight. Cells are incorporated in a low melting point agarose and, after lysis, they are subjected to an electric field (electrophoresis) that forces DNA to migrate out of the nucleoid towards the anode. When breaks are present in the DNA the molecule becomes more relaxed at that point, resulting in a more mobile DNA and making the cells look like a comet with a tail. After the electrophoresis, the gel is stained with a fluorescent dye and observed under a fluorescent microscope. The tail intensity is proportional to the number of DNA breaks. When compared to single-strand breaks the double-strand breaks increase the electrophoretic mobility due to a larger DNA fragmentation (Azevedo et al., 2011; Oliveira et al., 2012).

The tail length or the percentage of DNA in tail (tail intensity) can be quantified. Either by manual scoring or using imaging softwares (e.g. comet imager) that allow to measure the fluorescence intensity in the tail and converts it in a % of damaged DNA (Azqueta et al., 2011).

Although this assay was first described in 1984 by Östling and Johanson it is still most commonly used in cells without cell wall (mostly mammalian cells) and in some plant cells (Azevedo et al., 2010). In recent years comet protocols, have been adapted so that they can be used in yeast (Miloshev et al., 2002, Azevedo et al., 2010; Marques et al., 2011; Oliveira et al., 2012; Carvalho et al., 2016). The results were promising but not easily reproduced.

1.4.2 Real-Time PCR (RT-qPCR)

Real Time quantitative polymerase chain reaction (RT-qPCR) is a fast and accurate method that can be used to measure gene expression. It is used to amplify and simultaneously detect or quantify a targeted DNA molecule (Bustin et al., 2009).

Sample homogenization, RNA extraction, and cDNA synthesis can influence RT-qPCR results, so their variability must be controlled. In order to measure only the biological variation in samples, we have to annihilate technical variation. Reference genes must be used as internal controls, such as Alg9 and Taf10 (Teste et al., 2009; Dheda et al., 2005). The reference genes selected must be stable in the studied samples and show a strong correlation with the total amount of mRNA present in the samples (Bustin et al., 2009).

There are two methods that are used for product detection, a non-specific whereas a fluorescent dye intercalates with any double-stranded DNA (SYBR Green), and a specific whereas the DNA probes are labeled with a fluorescent reporter which allows us the detection after hybridization with the complementary mRNA (TaqMan® probes) (Pfaffl et al., 2009). If there are high levels of fluorescence it means that the sample has a high level of double-stranded DNA molecules (Corthell, 2014).

2. Objectives

2.1 Objectives

Human exposure to Microcystin-LR has become more frequent, as an increasingly growing need to use superficial waters from dams and water reservoirs. Microcystins have been an interesting subject of study for some decades. Since the World Health Organization (WHO) established a maximum value for microcystin in drinking water, contact to high concentrations of microcystin are becoming rare especially in developed countries. Thus, prolonged exposure to low doses tends to be problematic since it is asymptomatic and is reported to be potentially carcinogenic.

This project aims to characterize cytotoxic and genotoxic effects from exposure to low doses of microcystin over time using *S. cerevisiae* as a model system.

The specific objectives of this project are:

1. Asses the viability of *S. cerevisiae* VL3 cells exposed to different concentrations of MC-LR;
2. Evaluate the changes in expression levels of genes involved in DNA repair system (BER), in *S. cerevisiae* VL3 cells exposed to different concentrations of microcystin-LR;
3. Assess the level of DNA damage induced by microcystin-LR in *S. cerevisiae;*
4. Estimate the relation between cytotoxicity or genotoxicity and exposure to different microcystin concentrations.

3. Materials and Methods

3.1 *Saccharomyces cerevisiae* culture

The model organism used to perform the toxicity evaluation studies was *Saccharomyces cerevisiae*, mostly known as baker yeast. The strain used in this study was VL3, that is commercially available (Zymaflore®). The main stock used was lyophilized (Figure 3.3). To obtain viable cells in a suspension culture some grains of the *S. cerevisiae* VL3 lyophilized pre-culture were disposed in 20 mL YPD (yeast extract, peptone, dextrose) liquid medium (Annex I), in a 100 mL culture flask with a screw cap (Duran®). The culture was left growing overnight in a climatic chamber (FitoClima s600 Aralab®) at 20 ± 2ºC in a shaker (IKA Labortechnik®) (Figure 3.2).

Subsequently with the help of a sterilized inoculation loop some of the pre-inoculum was withdraw and plated in a solid YPD medium (Figure 3.1). The culture was stored in a refrigerator at 4ºC. The procedure was repeated within every three to four weeks to prevent using old *S.cerevisiae* cells to prepare the pre-inoculums.

All processes that involve handling cultures and growth mediums were performed under sterile conditions, in a laminar flow chamber (*Hera Safe*®).

Figure 3.2: *S.cerevisiae in YPD medium (Photograph by Sara Barreiros)*

Figure 3.1: Pre-inoculum *(Photograph by Sara Barreiros)*

Figure 3.3: *S. cerevisiae lyophilized (Photograph by Sara Barreiros)*

3.2 Microcystin-LR concentrations tested

A pure toxin standard solution was used to assure that the responses obtained were only due to MC-LR. The MC-LR 1 mg commercial stock solution (Enzo®) was diluted in PBS, so that the diluent would not cause additional damages to the cells, to a final concentration of 1 mg/mL. Four different concentrations of microcystin-LR were tested: 1 nM, 10 nM, 100 nM and 1 µM. The established guideline value for MC-LR in drinking water is 1 µg/L (1 nM) (WHO, 2008), therefore that concentration was chosen as the base value. According to previous studies, 1 µM of MC-LR is a concentration where a different cell response can be assessed (Valério et al., 2014). The tested solutions were obtained by diluting the stock solution. We kept and maintained the various dilutions in 1.5 mL flasks that were stored at -20ºC, in the laboratory freezer (ThermoFisher®).

3.3 Analysis of *Saccharomyces cerevisiae* viability when exposed to different concentrations of MC-LR

To evaluate cells viability when exposed to different chemical components and microcystin, MTT assays were performed. The MTT (methyl-thiazolyl-tetrazolium) stock solution (Sigma®) was diluted in PBS (phosphate buffered saline) (Invitrogen®) to obtain a final solution at 0.5 mg/mL. The MTT solution was stored and kept in the dark, given that the component is photosensitive, until it was used latter that day.

Saccharomyces cerevisiae VL3 pre-culture was left growing overnight in YPD medium at 20 ± 2ºC in a climatic chamber (FitoClima s600 Aralab®) with agitation. The following day, to perform the MTT assays, a six well plate (Nunc®) was used to inoculate 2 mL cultures with an initial optical density of 0.05, corresponding to approximately 5.5 x 10^5 cells/mL (OD660 vs Number of Cells, 2016 from http://www.pangloss.com/seidel/Protocols/ODvsCells.html, accessed on November 2016). The cultures were grown for 4 hours in the climatic chamber (FitoClima s600 Aralab®) at 20 ± 2ºC in a shaker (IKA Labortechnik®).

Two different compounds were used as positive controls: sodium dodecyl sulfate (SDS) (Invitrogen®) and hydrogen peroxide (H_2O_2) (Sigma-Aldrich®). SDS is a detergent commonly used to lyse cells and is expected to reduce cell viability. H_2O_2 causes damages to vital cellular components, such as mitochondria. Due to their different levels of toxicity when in contact with *S.cerevisiae* cells, each component had their own specific exposure time.

To perform the SDS positive control assays, two six well plates (Nunc®) were inoculated with 0.1%, 1% and 10% SDS and compared to the control (without SDS). Two different exposure times, one and two hours, were tested in order to evaluate the differences among the viability rate. The highest concentration used was 10% SDS, since previous studies showed that it drastically reduces culture viability (Chong et al., 2000).

The H_2O_2 positive control assay was also performed using a six well plate (Nunc®) that was inoculated with 3 mM, 6 mM and 12 mM H_2O_2 and compared to the control (no H_2O_2 added). The exposure time was only 30 min.

To perform the MTT assay cultures were exposed to different concentration of MC-LR, 0 nM (negative control), 1 nM, 10 nM, 100 nM and 1 µM for 4 h.

After the exposure to different components, 1 mL of the suspension of every well/flask was harvest to a 2 mL eppendorf and centrifuged for 5 min at 8.000 g (Eppendorf 5415C Centrifuge®). The supernatant was discarded and the cells were resuspended in 100 µL of PBS. Then 100 µL of MTT solution, previously prepared were added, and the eppendorfs incubated in the dark in the climatic chamber with agitation for 2 h. Afterwards the suspension was centrifuged for 5 min. at 8.000 g and the supernatant was discarded. The pellet was resuspended in 300 µL DMSO (ThermoFisher®) and 100 µL of each treatment was inoculated in a 96 well plate (Starstedt®), performing in total 3 replicates. The wells absorbance was read by a spectrophotometer (ThermoFisher Labsystems®) at 570 nm with a reference wavelength of 690 nm. The protocol is described in more detail in Annex II.

3.4 Analysis of *Saccharomyces cerevisiae* genotoxic effects when exposed to different concentrations of MC-LR

To access *S. cerevisiae* genotoxic effects when exposed to MC-LR, particularly DNA damage, the Comet assay was performed. Given that there is very limited information about the YCA, the method reported by Oliveira and Johansson (2012) was primarily adopted. Several modifications were sequentially made along the assays conducted to try to get reproducible results.

The yeast culture preparation was similar to the one used for the MTT assay, *S. cerevisiae* VL3 pre-culture was incubated overnight in YPD medium in a climatic chamber (FitoClima s600 Aralab®). The following day, to perform the Comet assay, six well plates (Nunc®) were used to inoculate 5 mL cultures with a cell density adjusted to an initial optical density of 0.05, corresponding to approximately 5.5 x 10^5 cells/mL (OD_{660} *vs* Number of Cells, 2016 from http://www.pangloss.com/seidel/Protocols/ODvsCells.html, accessed on November 2016). The cultures were grown for 4 h at the climatic chamber (FitoClima s600 Aralab®) at 20 ± 2ºC in a shaker (IKA Labortechnik®).

H_2O_2 (Sigma-Aldrich®) was used as a positive control, since it causes DNA damage that is detected by the Comet Assay. Cells were exposed for 30 min to this positive control, taking into account previous results (Valério et al, 2014).

Cells were harvested in 2 mL eppendorf by centrifugation at 2.000 g for 2 min and washed twice with 1 mL of ice-cold deionised water. Afterwards cells were resuspended in 2 mg/mL of lyticase (Sigma®) previously diluted in S-Buffer (1 M sorbitol and 25 mM KH_2PO_4, pH=6.5) and incubated at 35 °C for 30 min at 500 rpm, in order to obtain spheroplasts.

Spheroplasts were collected by centrifugation at 2.000 g for 2 min, twice with 100 µL ice-cold S-buffer, and then resuspended with S-buffer so that in the end the pellet had a volume of 60 µL.

The pellet was suspended in 100 µL of low melting agarose (1.5 % by mass per volume in S-buffer) at 35 °C.

The pellet, 40 µL, was disposed in a glass slide previously coated with 1% agarose, and then a coverslip was placed on each gel. After removing the coverslips, the glass slides were transferred to lysis solution (4ºC) for 30 min, to lyse the spheroplasts. Afterwards the slides were washed with neutralization buffer (4ºC) for 5 min, twice, to remove lysis solution. Finally, the glass slides were placed in a tank with electrophoresis buffer (4ºC) for 20 min, so that the DNA could unwind. And then submitted to electrophoresis, for 10 min.

After removing the slides from the electrophoresis tank, these were placed in a neutralization buffer (4ºC) for 10 min and kept at room temperature in the dark for 2 to 3 days, in order to dehydrate agarose. Subsequently, the gels on the glass slides were stained with 36 µL of GelRed™ (Biotium®) diluted (1:10000) in water.

At least 50 comet images were acquired from each sample, with a fluorescence microscope (Axioplan 2, Zeiss) using the Comet Imager 2.2 (MetaSystems) with a magnification of 630x.

In Table 3.1 it is summarized all different modifications done to the Oliveira and Johansson (2012) method, including duration of the electrophoresis and voltage as well as solutions compositions. A more detailed protocol is described in Annex III.

Table 3.1: *Comet assay reagents composition*

Stock solution	Components (mM)	pH	Exposure time tested (min)
Lysis solution (1)	NaOH (30 mM)	12	30; 60
	NaCl (1 M)		
	Tris-HCl (10 mM)		
	EDTA (50 mM)		
	N-laurylsarcosine (0.1% w/v)		
Lysis solution (2)	NaOH (30 mM)	12	30
	NaCl (1 M)		
	Tris-HCl (10 mM)		
	EDTA (50 mM)		
	Triton-X-100 (1% v/v)		
Electrophoresis buffer (1)	NaOH (30 mM)	10	10; 20
	Tris-HCl (10 mM)		
	EDTA (10 mM)		
Electrophoresis buffer (2)	NaOH (300 mM)	10	20; 40
	NaCl (1 M)		
	N-laurylsarcosine (0.05% w/v)		
	Tris-HCl (10 mM)		
	EDTA (1 mM)		
Electrophoresis buffer (3)	NaOH (300 mM)	13	20; 40
	NaCl (1 M)		
	N-laurylsarcosine (0.05% w/v)		
	EDTA (1 mM)		
Neutralization buffer	Tris-HCL (10 mM)	7.4	2 x 5
S-buffer	Sorbitol (1 M)	6.5	-
	Monopotassium phosphate (25 mM)		

Legend: *For the lysis buffer, the neutralization buffer and S-buffer the components were added to 250 mL of deionized water; For the electrophoresis buffer the components were added to 2 L of deionized water*

YCA - Trial 1. Lyticase only acted for 5 min, at room temperature without shaking, and the lysis solution 1 (see annex III), was applied during 20 min. The glass slides were washed three times for 20 min. with electrophoresis buffer 1 (see annex III). The electrophoresis run during 15 min at 30 mA.

The neutralization buffer acted for 10 min and afterwards the glass slides were put in water for 10 min and latter kept in the dark at room temperature.

YCA - Trial 2. The same protocol used in YCA trial 1, except that the lyticase acted for 20 min and the glass slides were washed for 20 min with electrophoresis buffer 1 and the electrophoresis run was 10 min. at 30 mA. These changes were based on the protocol from Oliveira & Johansson, 2012.

YCA – Trial 3. Cells were exposed to 10 mM and 50 mM of H_2O_2. These drastic concentrations were chosen because the main goal was yet to be achieved, cell damage. This assay suffered several protocol changes such as the lyticase, which acted for 30 min with shaking in the Thermoblock at 30ºC. The glass slides were placed in an electrophoresis tank, filled with electrophoresis buffer, where they remained for 20 min and 40 min, since this step is critical to unwind cell DNA. The electrophoresis was run for 10 min at 26 mA for the glass slides with a 20 min unwinding time and 20 min at 32 mA for the glass slides with a 40 min unwinding time. The glass slides, were washed twice in neutralization buffer for 5 min and afterwards with distilled water.

YCA – Trial 4. Cells were exposed to the same H_2O_2 concentrations as above, 10 mM and 50 mM. Since no significant difference was observed between a 20 min and a 40 min unwinding (Figure 4.6), the glass slides were placed in electrophoresis tank where they remain for 20 min with the respective electrophoresis buffer 2 or electrophoresis buffer 3, that are further described in annex III.

YCA – Trial 5. Since the electrophoresis buffer, used in this assay, is more aggressive for the cells, it was decided to lower the peroxide concentration from 50 mM to 25 mM for fear that the damage would be higher and would not be representative. After the lyticase has acted, an additional pre-treatment with 10 mM of H_2O_2 was applied to the samples, a method that was described by Staneva et al., 2013. This allows to establish a new baseline for DNA damage. The new lysis buffer 2 was used, which is described in annex III. The electrophoresis buffer 2 was chosen for this test.

YCA – Trial 6. In this experiment the gel was made with a 1.5% and a 2.5% of low melting agarose. The cells were exposed to the same conditions as the fifth assay, 10 mM of H_2O_2 and 25 mM of H_2O_2 plus an additional pre-treatment to 10 mM H_2O_2 on each sample after the cell wall degradation.

YCA-Trial 7. In this assay the conditions used were the same as the trial 6, except for the time of electrophoresis run, which was 10, 20 and 30 min.

3.5 *Saccharomyces cerevisiae* nucleic acid extraction

3.5.1 DNA extraction

To optimize PCR conditions for the various selected primers it was necessary to extract DNA from *S. cerevisiae*. This DNA was used as a positive control in conventional PCR, when checking if RNA was well purified. Furthermore, several dilutions of the DNA were prepared to make a calibration curve for Real-Time PCR (RT-qPCR). The DNA protocol extraction (Annex IV) was performed using a DNA extraction kit; Spin Plant Mini Kit (Invitek®).

After performing the DNA extraction protocol, the DNA concentration and quality were determined using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific).

3.5.2 RNA extraction and purification

To evaluate the alterations of the expression levels of the *S. cerevisiae* selected genes by RT-qPCR, *S.cerevisiae* RNA had to be extracted and purified. *S. cerevisiae* VL3 pre-culture was left growing overnight in YPD medium in a climatic chamber (FitoClima s600 Aralab®). The following day, 20 mL culture was inoculated in five 150 mL flasks with a cell density adjusted to an initial optical density of 0.05, corresponding to approximately 5,5 x 10^5 cells/mL. Each flask was treated with different MC-LR concentrations, 0 nM (control), 1 nM, 10 nM, 100 nM and 1 µM. The cultures were left growing for 4 hours at the climatic chamber (FitoClima s600 Aralab®) at 20 ± 2ºC in a shaker (IKA Labortechnik®).

Cells were harvest in 15 mL plastic tubes (Sarstedt®) by centrifugation at 834 g on a high capacity centrifuge (Sorvall® RT 6000D) for 3 min and washed with 1 mL of RNase free sterile water (Gibco®).

Cells were transferred into a 1.5 mL eppendorf and centrifuged (Eppendorf 5415C Centrifuge®) for 3 min at 8.000 g. The supernatant was discard and the pellet was immediately freezed in liquid nitrogen and keep at -80ºC until it was used (for more details see Annex IV).

Afterwards RNA samples were purified using the High Pure RNA Isolation Kit (Roche®), according to the manufacturer instructions (Annex V).

Finally, RNA concentration was measured by spectrophotometry, NanoDrop™ (Thermo Scientific®) and their purity was accessed (Oxford Gene Technology, 2017 from http://www.ogt.co.uk/resources/literature/483_understanding_and_measuring_variations_in_dna_sample_quality, accessed on June 2016).

To confirm that the purified RNAs were indeed free from DNA, the samples were subjected to a conventional PCR protocol (Annex VII).

3.6 *Saccharomyces cerevisiae* studied genes and primers design

In this study, RT-qPCR will be used to provide quantitative measurements of gene expression. It intends to determine how the expression of a particular gene changes in response to microcystin exposure. Knowing that MC-LR causes oxidative DNA damage that is mainly repaired by the *S. cerevisiae* DNA BER system, the following genes were selected: *Rad27*, *Apn1*, *Apn2*, *Ntg1* and *Ntg2* to evaluate their expression levels following cells exposure to microcystin. *Ntg1* and *Ntg2* genes were previously briefly analyzed by Valerio et al. 2016, but we decided to include them in the study where more conditions were tested. Also, the expression levels of *Cdc55* gene, that is associated with protein phosphatase PP2A were evaluated. The RT-qPCR technique was used to quantify the expression level of these genes.

RT-qPCR technique measures the quantity of mRNA that was produced during the transcription of a specific gene (Stephenson, 2010). Since the applied method is based on the relative quantification

of mRNA, we must have reference genes. These genes, also known as housekeeping genes, have a constant expression under normal and patho-physiological conditions, since they are essential for the maintenance of basic cellular function (Eisenberg and Levanon, 2003). We selected two reference genes *Taf10* and *Alg9*, which are described below in Table 3.2.

Table 3.2: *Description of reference genes and their functions*

Gene	Primer sequence	Molecular Function	Reference
Alg9	F: 5'-CACGGATAGTGGCTTTGGTGAACAATTAC-3'	Mannosyltransferase activity/Protein amino acid glycosylation	Teste et al. (2009); Nadai et al. (2015)
	R: 5'-TATGATTATCTGGCAGCAGGAAAGAACTTGGG-3'		
Taf10	F: 5'-ATATTCCAGGATCAGGTCTTCCGTAGC-3'	RNA Pol II transcription factor activity/Transcription initiation and chromatin modification	Teste et al. (2009); Nadai et al. (2015)
	R: 5'-GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG-3'		

The primers selected for the genes under study were designed according to several characteristics, ideal for RT-qPCR: the length of the primer had to be between 18 and 25 bp, the melting temperature had to be between 53ºC and 63ºC and the length of the product had to be between 100 and 300 bp The process is described in detail in Annex VIII. The selected primers are described in the table below (Table 3.3).

Table 3.3: *Description of primers for BER and Cdc55 genes and their functions*

Gene	Primer sequence	Molecular Function	Reference
Rad27	F: 5'- CCG CAG CAA GTG AAG ATA TG - 3'	5'-flap endonuclease (Kao et al., 2002)	This study
	R: 5'- CCA ACA CCT CTG ATG CTT TC - 3'		
Apn1	F: 5'- TGG GTT TCT CCG CAG TAT - 3'	AP (apurinic/apyrimidinic) endonuclease and 3'-repair diesterase (Jilani et al., 2003)	This study
	R: 5'- GCC TAT CCC TAA TTG CTC AC - 3'		
Apn2	F: 5'- TGC TAA TGG GCG ACG TAA AT - 3'	AP endonuclease (Unk et al., 2000)	This study
	R: 5'- GGC GTG TCC GGA TTG ATA ATA - 3'		
Ntg1	F: 5' - CAT TCC TGT AAC GGT TGC CT - 3'	DNA N-glycosylase and AP lyase (Meadows et al., 2003)	Valério et al., 2016
	R: 5'- TTG TGT GGA ACC CAA CTG AA - 3'		
Ntg2	F: 5'- AAC ACT GCA AAA AGG TTG GG - 3'	DNA N-glycosylase and AP lyase (Meadows et al., 2003)	Valério et al., 2016
	R: 5'- GAC CAA ATC CAA CCA AAA CG - 3'		
Cdc55	F: 5'- ACA GGC ATG GTG GGA AGT AG - 3'	Regulatory subunit B of protein phosphatase 2A (PP2A) (Hombauer et al., 2007)	Valério et al., 2016
	R: 5'- CAA TAT CGT CAC CCC AAT CC - 3'		

The primers of the following genes *Cdc55*, *Ntg1*, *Ntg2*, had been previosly designed by Valério et al. (2016a). However, the primers for the following genes, *Rad27*, *Apn1* and *Apn2* had to be designed in this study. The genes sequences were extracted from SGD (Saccharomyces Genome Database, 2016 from http://www.yeastgenome.org/, accessed on May 2016) and then uploded on Primer3 tool from simgene (Simgene, 2016 from http://www.simgene.com/, accessed on February 2016) which allowed to design each pair of primers (Annex VIII). This procedure allowed to choose the best primer pairs, that were latter synthesized by ThermoFisher Scientific®.

The annealing sites for primers of *Apn1*, *Apn2*, *Rad27*, *Ntg1*, *Ntg2* and *Cdc55* genes are illustrated below, in Figure 3.4.

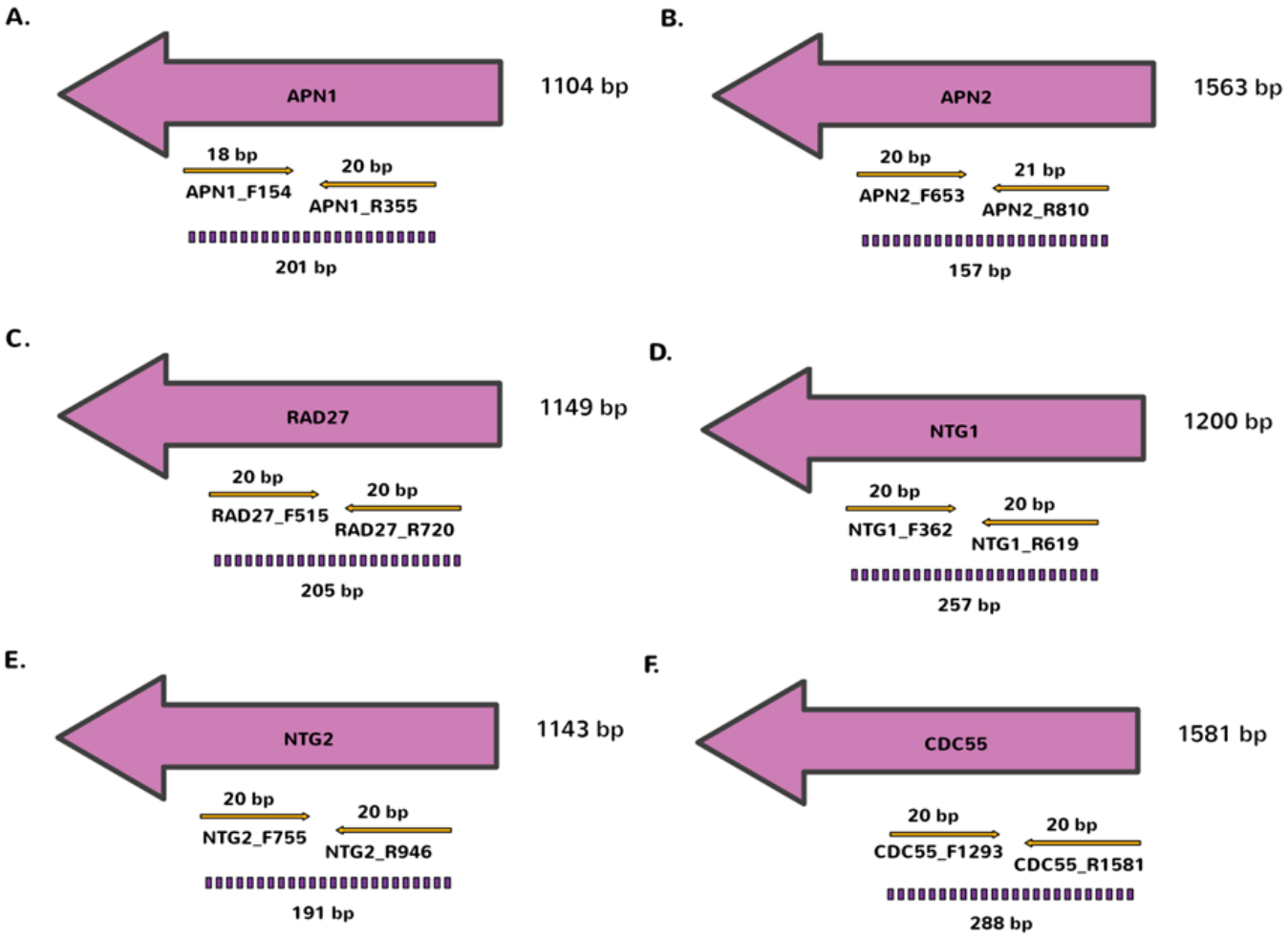


Figure 3.4: *Annealing sites for the following genes A) Apn1; B) Apn2; C) Rad27; D) Ntg1; E) Ntg2; F) Cdc55*

3.6.1 Conventional PCR parameters

After selecting the primers it was mandatory to verify if they would indeed amplify the target fragment of *S.cerevisiae* DNA and to optimize PCR conditions, especially the annealing temperature. A conventional PCR was preformed, as exemplified, (Figure 3.5), for each sample, allowing to outwit any DNA or RNA contamination.

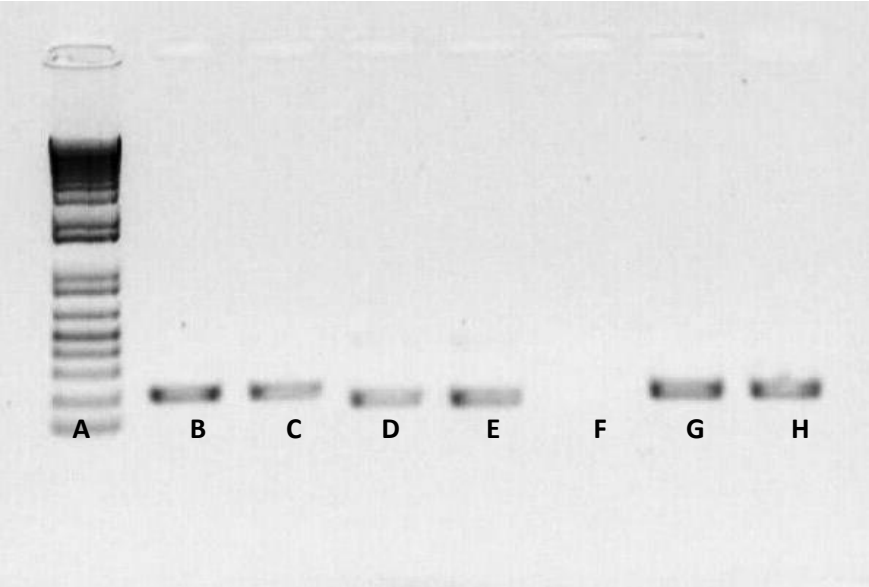


Figure 3.5: *Conventional PCR - A) DNA ladder; amplification of: B, C) Apn1 gene (201 b.p.) D, E) Apn2 gene (157 b.p. F) negative control (Apn1 primers, without DNA); G, H) Rad27 gene (205 b.p.). (Image from Sara Barreiros)*

The conventional PCR was preformed using a mix of the following reagents; 15.55 µL of RNase and DNase free water (Gibco®); 2.5 µL of 1x PCR Rxn buffer (Invitrogen®); 0.5 µL of dNTPs (Invitrogen®); 1.25 µL of each primer (50 µM), forward and reverse (Thermo Fisher Scientific®); 1.25 µL of 1% (v/v) W-1 (Invitrogen®); 1.5 µL of $MgCl_2$ (Invitrogen®); 0.2 µL of Taq DNA Polimerase (Invitrogen®); and 1 µL of DNA 100 ng/µL (for each respective PCR tube) for anneling temperature optimization or 1 µL of RNA 100 ng/µL (for each respective PCR tube) to outwit any DNA contamination. The amplification was made usig a thermocycler TGradient (Biometra®) with the following programme (Table 3.4). The PCR products were analyzed by electrophoresis with a 1% agarose gel made with 1 µL of GelRed Nucleotic Acid Strain (Biotium®). Conventional PCR protocol description in annex (Annex IX).

Table 3.4: *Conventional PCR program for Apn1, Apn2 and Rad27 primers*

Step	Temperature (ºC)	Time	Nº of cycles
Initial Denaturation	94	5 min	1 x
Denaturation	94	45 sec	35 x
Annealing	50 /51(*)	45 sec	
Extension	72	45 sec	
Final Extension	72	5 min	1 x

Legend: (*) Annealing temperature for *Apn1* 50ºC; Annealing temperature for *Apn2* and *Rad27* 51ºC

3.6.2 Calibration curves for RT-qPCR

Before starting the RT-qPCR assays to measure the expression levels, the optimum conditions asserted for conventional PCR had to be verified if they would also be the optimal condicions used in

RT-qPCR. The linear regression coefficient (R^2) must be above 0.95% and efficiency of the process must be betwen 80-115% (Stephenson, 2010). The optimization method is based on the construction of standard calibration curves.

First the DNA, previously quantified with NanoDrop 1000 (Thermo Scientific®), was diluted to obtain different concentration of DNA, 100 ng/uL; 10 ng/uL; 2 ng/uL and 1 ng/uL. The different dillutions were used in RT-qPCR using Rotor-Gene 3000 (Quiagen-Corbett®) for *Apn1*, *Apn2* and *Rad27* set of primers, and the kit SensiMix™ SYBR No-ROX (BioLine®), the process and the quantities used are described in "RTq-PCR program parametres" above and in the table 3.4. The samples were analysed in triplicate and the amplification program was simillar to the one in table 3.4, however to choose the best anneling temperature each gene was tested at three different temperatures 48º; 50º and 52ºC. To confirm the specificity of the amplified PCR products, a melting curve was performed at the end of the 40 cycles with a gradual increase of temperature of 1ºC/second, from 50ºC to 95ºC. The threshold was fixed at 0.02 of fluorescence signal for every RT-qPCR, using Rotor-Gene 3000 series software.

3.6.3 RT-qPCR program parameters

The Real-Time qPCR techique synthesizes cDNA from RNA, allowing the measurement of transcipted RNA. The method used in this project will be non-specific since the probe used is SYBR green, which binds with any double-strand DNA.

All the RT-qPCR assays were preformed on Rotor-Gene 3000 (Quiagen-Corbett®). The SensiFAST™ SYBR no-ROX One-Step Kit (Bioline®) was used to preform the various assays. A mix was made with the following reagents of the kit (Table 3.5).

Table 3.5: *Master mix that are prepared for RT-qPCR*

Stock solutions	Volume per tube (µL)
DEPC-H_2O	0.20
MasterMix	5.00
Reverse Transcriptase	0.10
Forward primer	0.25
Reversed primer	0.25
10U/µL RiboSafe RNase Inhibitor	0.20
Final Volume	**6.00**

To the final volume of 6 µL it was added 4 µL of the corresponding RNA with a concentration of 10 ng/µL. All samples were analysed in triplicates.

The RT-qPCR programme settings for the *Apn1*, *Apn2* and *Rad27* genes are described bellow in Table 3.6.

Table 3.6: *RT-qPCR programme for Apn1, Apn2 and Rad27*

Step	Temperature (ºC)	Time	Nº of cycles
Reverse transcription	42	10 min	1x
Polymerase activation	95	10 min	1x
Denaturation	95	15 sec	40x
Annealing	variable (see details below)	30 sec	
Extension	72	30 sec	
Melting	50-95	Start with a 1ºC/sec ramp	1x

The annealing temperature was specific for each gene.

The annealing temperature for each primer reversed and forward was ascertaining by the following equation 3.1 (Von Ahsen et al., 1999 from http://www.biophp.org/minitools/melting_temperature/demo.php?formula=basic, accessed in April 2016):

$$\text{Tmelting } (^0\text{C}) = 64.9 + 41 * \frac{(\text{yG} + \text{zC} - 16.4)}{(\text{wA} + \text{xT} + \text{yG} + \text{zC})}$$

Where the y is the number of guanines (G), z is the number of cytokines (C), w the number of adenines (A) and x the number of thymines (T) of the respective primer.

For the reference genes, Alg9 and Taf10, the annealing temperature is 58ºC. For Ntg1 and Ntg2 genes, the annealing temperature is 48ºC. The Cdc55 gene, from PP2A, has an annealing temperature of 49ºC.

To confirm the specificity of the amplified product a melting curve was performed after 40 cycles, where the reaction has enter the plateu phase. This allowed to confirm that there was only one amplification product (only one peak was generated).

The intersection between an amplification curve and a threshold line (where the signal can be discriminated from background noise), is referred as the cycle threshold, CT value (Stephenson, 2010). The threshold line was set manualy to 0.02 in all the genes.

3.7 Gene Expression

3.7.1 Evaluation of reference genes stability

The evaluation of reference genes stability, *Taf10* and *Alg9*, was asserted previously by Teste et al (2009), where they verified that their expression remained stable, independently of growth conditions and strain backgrounds (Teste et al., 2009).

3.7.2 Quantification of gene expression

The relative quantitation of gene expression was based on the comparison of the expression levels of target genes with reference genes.

In this study the mathematic method of Pfaffl (2001) of relative quantification for RT-qPCR was used. This method is reproducible and reliable, and it does not require that the reference genes and targets reaction efficiencies to be equal, since the equation 3.2 performs the necessary corrections.

$$R = \frac{(E\ Target\ gene)^{\triangle Ct\ target\ gene\ (control-sample)}}{(E\ Reference\ gene)^{\triangle Ct\ reference\ gene\ (control-sample)}}$$

The letter "R" represents the expression rate of the target gene relative to the reference gene; "E target gene" represents the efficiency of RT-qPCR of target genes; "E reference gene" represents the efficiency of RT-qPCR of the selected reference gene; "∆CT target gene" represents the variation of the threshold cycle of the target gene (CT of the control condition – CT of the sample); "∆CT reference gene" represents the variation of the threshold cycle of the reference gene (CT of the control condition – CT of the sample of the reference gene to analyze). The value of the relative expression results of a medium of three measured values. All data was analyzed using the software program Microsoft Office Excel®.

4. Results

4.1 Viability assays

4.1.1 MTT Controls

Two different chemical compounds were used as positive controls in the MTT assay, to verify if this test was working correctly in *S. cerevisiae* cells.

The first positive control used was SDS (Sodium Dodecyl Sulfate), and cells were exposed during one or two hour to different concentrations of this detergent (0.1%, 1%, 10%). It can be seen that the time factor, 2 h exposure does not considerably induces alterations in the cells viability (Figure 4.1). A concentration-response relationship was observed, irrespectively of the treatment duration. It is clear that 1% SDS causes a decrease of viability by 50%, when compared with the control, which diminishes to 10% of viability with the highest SDS concentration tested (10% SDS). It can be observed that 0.1% SDS causes an increase in viability, this must be an artifact, a possible explanation might be the fact that there must be some level of solubilization among some cellular components that will later interfere with the test.

After applying the t-test to all tested conditions, all SDS results showed a significant concentration-response relationship, irrespectively of the treatment duration ($p < 0.05$), as we can see below in Figure 4.1.

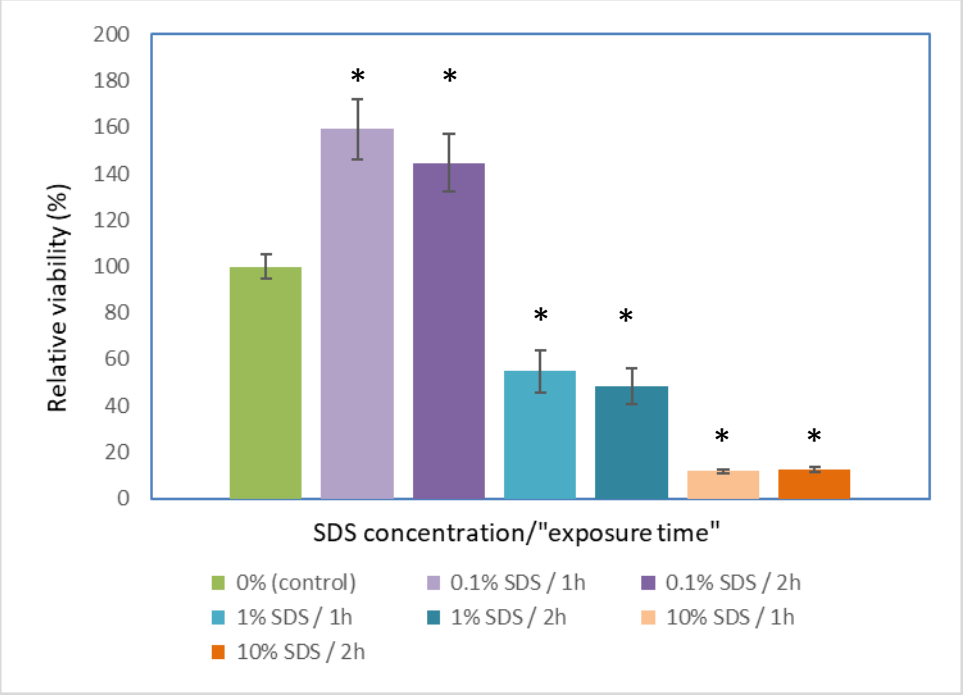


Figure 4.1: *S. cerevisiae VL3 viability (relative do control, %) after exposure to different concentrations of SDS: 0.1%, 1%, 10% for two different exposure times: 1h and 2h (average of 3 biological replicas)*

The second positive control used was H_2O_2 (hydrogen peroxide) at different concentrations (3 mM, 6 mM and 12 mM) to which the cells were exposed for 30 min. It can be seen that 3 mM of H_2O_2 reduces viability by 10%, 6 mM of H_2O_2 reduces viability by 20% and 12 mM of H_2O_2 reduces viability by 40%. Although there is a trend towards lowering cell viability with increasing peroxide concentration,

only the cells exposed to 12 mM of H_2O_2 displayed significant differences from control ($p < 0.05$), as we can see in Figure 4.2.

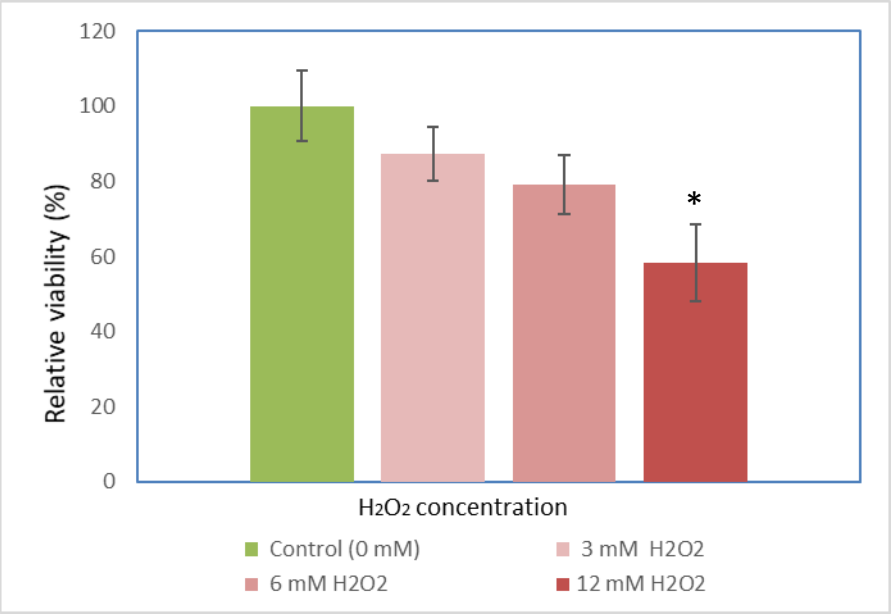


Figure 4.2: *S. cerevisiae exposed to different concentrations of H2O2 3 mM, 6 mM and 12 mM (average of 3 biological replicas) * denotes a statistically significant difference between the treatment and the control cells (p < 0.05)*

4.1.2 *Saccharomyces cerevisiae* viability when exposed to different concentrations of MC-LR

After confirming that the assay was working properly with *S.cerevisiae* VL3 cells, given the results obtained when exposing the cells to different positive controls, the assay was applied to cells that had been exposed to MC-LR. The cells had a 4-hour exposure to different concentrations of microcystin: 1 nM, 10 nM, 100 nM and 1 µM. It can be seen that 1 nM of MC-LR increases viability by 10%, 10 nM increases viability by 20%, 100 nM increases viability by 15% and 1 µM of MC-LR increases viability by 20%.

Although there is a trend towards observing a higher cell viability when exposing cells to higher microcystin concentrations, only two of the concentrations studied (10 nM and 1 µM of microcystin) showed a statistically significant difference between the treated and the control cells ($p < 0.05$), as we can see in Figure 4.3. The results obtained from MTT assay with MC-LR differ widely from what was expected, since exposure to microcystin was hoped to decrease cell viability.

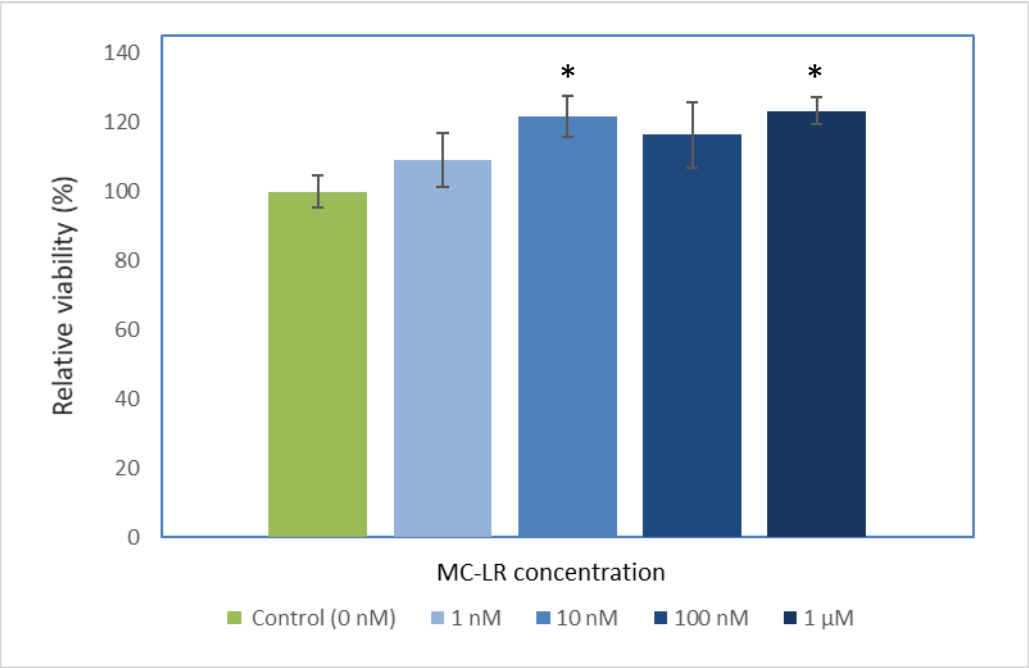


Figure 4.3: *S. cerevisiae exposed to different concentrations of microcystin 1 nM, 10 nM, 100 nM and 1 µM. The results presented on the chart are the media of the triplicate replicas of the assay, as well as their standard deviation (average of 3 biological replicas).* denotes a statistically significant difference between the treated and the control cells ($p < 0.05$)*

4.2 Comet Assay

4.2.1 Optimization of assay parameters

The implementation of the YCA started by exposing cells to different concentrations of hydrogen peroxide, a widely used positive control for the Comet assay.

YCA - Trial 1. In the first assay yeast cells were exposed to 3 mM, 5 mM and 25 mM of H_2O_2. These concentrations were chosen because previous studies had shown that DNA lesions were induced in yeast cells with 10 mM H_2O_2 (Ribeiro et al., 2006) and some previous results (Valério et al, 2014). Therefore, slight damages with 3 mM and 5 mM, and severe damages with the higher concentration of 25 mM were expected to be observed. The protocol followed is described in annex III and is an adaptation of Oliveira and Johansson (2012).

Figure 4.4 represents cells that were exposed to 3 mM of H_2O_2 in YCM-trial 1. The other concentrations of the positive control, 5 mM and 25 mM, did not result in visually different images, which may be due to the fact that some components such as lyticase, which degrades the cell wall, did not act correctly or during the necessary time. The assay was repeated twice to confirm the previous results (not presented in the thesis), but it showed no significant differences from the results showed in Figure 4.4.

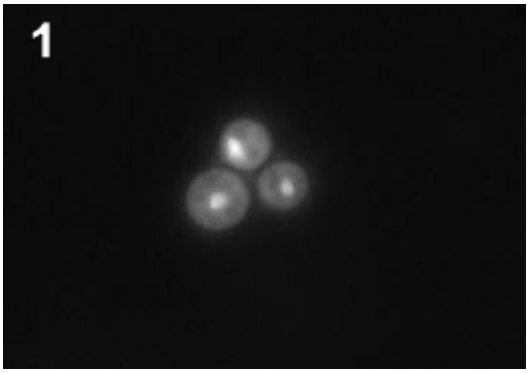


Figure 4.4: *Comet assay #1; S. cerevisiae cells exposed to 3 mM of hydrogen peroxide (magnification 1000x)*

In the second assay, YCA-Trial 2, cells were exposed to the same conditions as in Trial 1, the goal of this assay was to correct the exposure time and solutions used in the first assay.

Since no significant differences were observed in cells exposed to 3 mM, 5 mM and 25 mM of H_2O_2, the hydrogen peroxide concentration used in the following assays was drastically increased, to obtain cell damage. The Figure 4.5 represents cells that were exposed to 25 mM (1) and 50 mM (2) of hydrogen peroxide, respectively. The cells exposed to 3 mM H_2O_2, were not significantly different from the images presented in figure 4.5. It can be seen, that the DNA is still inside the nucleus because the cell wall is still intact, therefore there is no DNA migration and consequently no comet tail is formed. The assay problem seems to be related with cell wall degradation. Similar to the first assay, this one was also repeated (results not presented), but with no significant differences from the results showed in Figure 4.5.

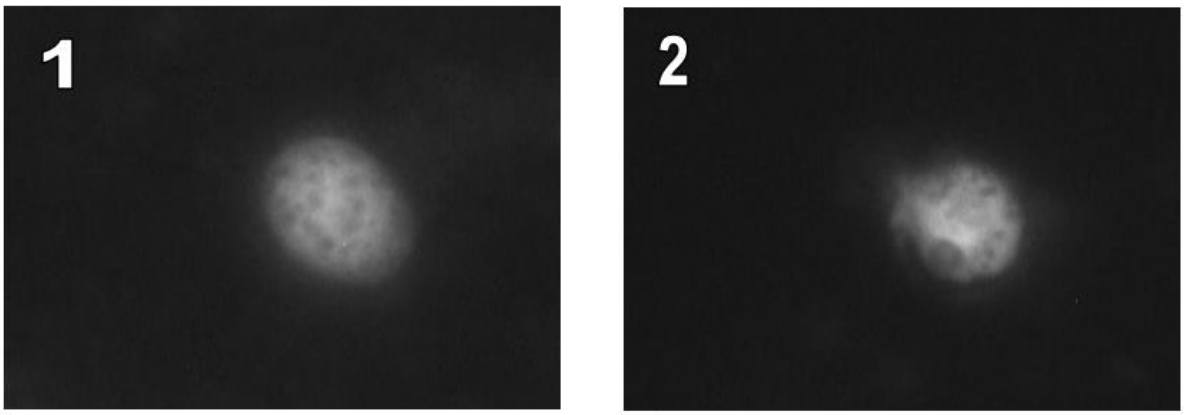


Figure 4.5: *Comet assay #2; S. cerevisiae cells exposed to 25 mM (1) and 50 mM (2) of hydrogen peroxide (amplification 1000x)*

The results of YCA – Trial 3 are represented in Figure 4.6, which represents control cells (1), cells that were exposed to 50 mM of H_2O_2 (2), with a 20 min unwinding and 10 min electrophoresis, and control cells (3), cells that were exposed to 10 mM of H_2O_2 (4), with a 40 min unwinding and 20 min electrophoresis. There was no differentiating feature between cells exposed to different H_2O_2 concentrations, as it can be seen in the representative images in Figure 4.6. Although no significant difference could be observed between the different hydrogen peroxide concentrations and electrophoresis parameters, it seems that the treatment had some positive results since in image 2, 3 and

4 of Figure 4.6 the cell's DNA seems to be relaxed and extruded from the nucleus. However, there was no tail formation.

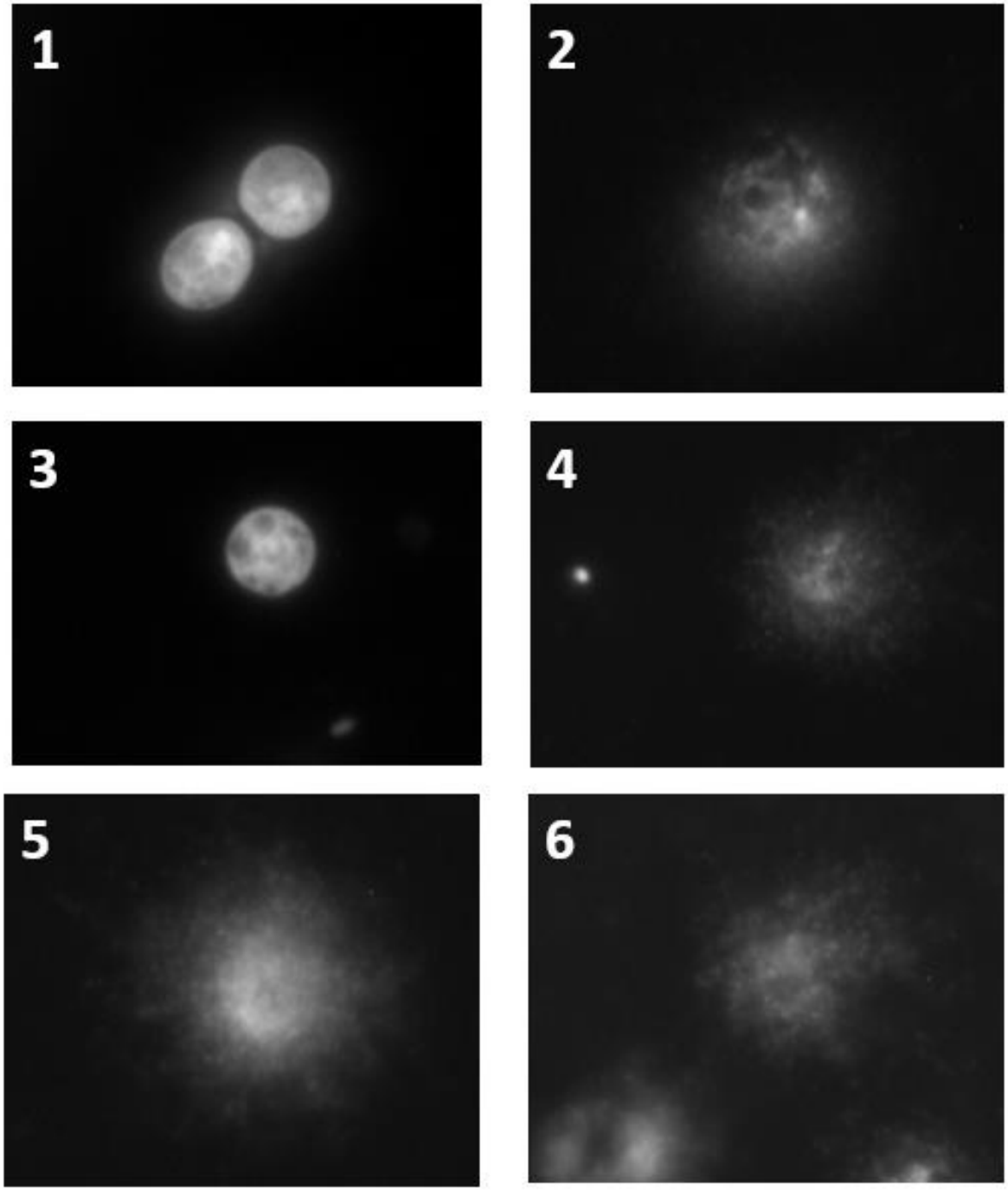


Figure 4.6: *Comet assay #3; S. cerevisiae control cells with 20 min unwinding and 10 min of electrophoresis (1); and with with 40 min unwinding and 20 min of electrophoresis (2); S. cerevisiae exposed to 10 mM of H_2O_2 with 20 min unwinding and 10 min of electrophoresis (3); and with 40 min unwinding and 20 min of electrophoresis (4); S. cerevisiae exposed to 50 mM of H_2O_2 with 20 min unwinding and 10 min of electrophoresis (5); and with 40 min unwinding and 20 min of electrophoresis (6) (amplification 1000x)*

YCA – trial 4. In this forth assay the goal was to determine which electrophoresis buffer, 2 or 3, was the most adequate. Since there was no difference observed in electrophoresis running time, the electrophoresis run was kept for 10 min at 106 mA for the glass slides that were submerge in electrophoresis buffer 2 and 10 min at 320 mA for the glass slides that were submerged in

electrophoresis buffer 3. The different electrophoresis buffer compositions and their pH have influence on the current and amperage of the electrophoresis, which is why the value changes greatly.

The Figure 4.7 represents cells that were exposed to 50 mM of H_2O_2 and to electrophoresis buffer 3 (1) and control cells that were exposed to electrophoresis buffer 2 (2). The cells that were exposed to different concentrations from the ones here presented were not significantly different from the images shown in Figure 4.7. In the assay, something must have gone wrong since there seems that there was no effective cell lysis, which means that no conclusion can be draw regarding the most suitable electrophoresis buffer. The assay was repeated twice (results not presented), but it showed no significant differences from the results showed in Figure 4.7.

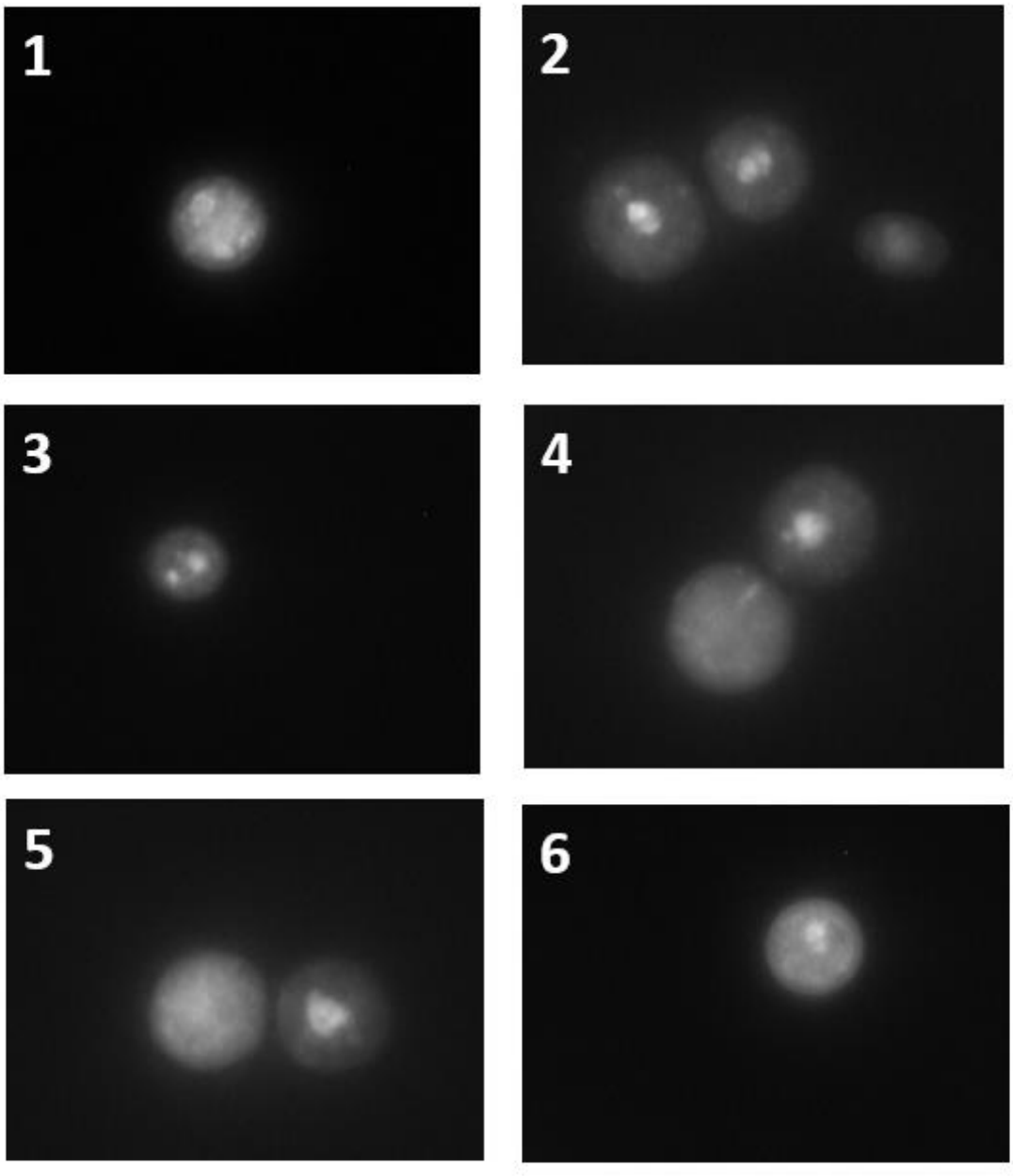


Figure 4.7: *Comet assay #4; S. cerevisiae control cells with electrophoresis buffer 3 (1); and with electrophoresis buffer 2 (2); S. cerevisiae exposed to 10 mM of H_2O_2 with electrophoresis buffer 3 (3); and with electrophoresis buffer 2 (4); S. cerevisiae exposed to 50 mM of H_2O_2 with electrophoresis buffer 3 (5); and with electrophoresis buffer 2 (6) (amplification 1000x)*

YCA-Trial 5. After the forth assay showed no results regarding cell lysis, the goal of the fifth assay was to test and verify if a new lysis buffer was more effective in destroying the cell wall. In this assay the cells were exposed to a positive control of 10 mM and 25 mM of H_2O_2.

The Figure 4.8 represents control cells (1) and cells that were exposed to 25 mM of H_2O_2 (2) both were exposed to an additional pre-treatment with 10 mM of H_2O_2. The cells that were exposed to the different H_2O_2 concentrations showed a considerable difference in cell degradation when compared to the control, images of cells exposed to 10 mM of H_2O_2 where not showed, because the results were similar to the image 2 (cells exposed to 25 mM of hydrogen peroxide) of Figure 4.8. It can be seen on Figure 4.8 that the DNA is exposed, which means that the new lysis buffer was more adequate for the assay. However, there is no comet tail formation, which may be due to the electrophoresis run conditions. This assay was also repeated twice, to confirm the results obtained, (results not presented in the thesis), but it showed no significant difference from the images in Figure 4.8.

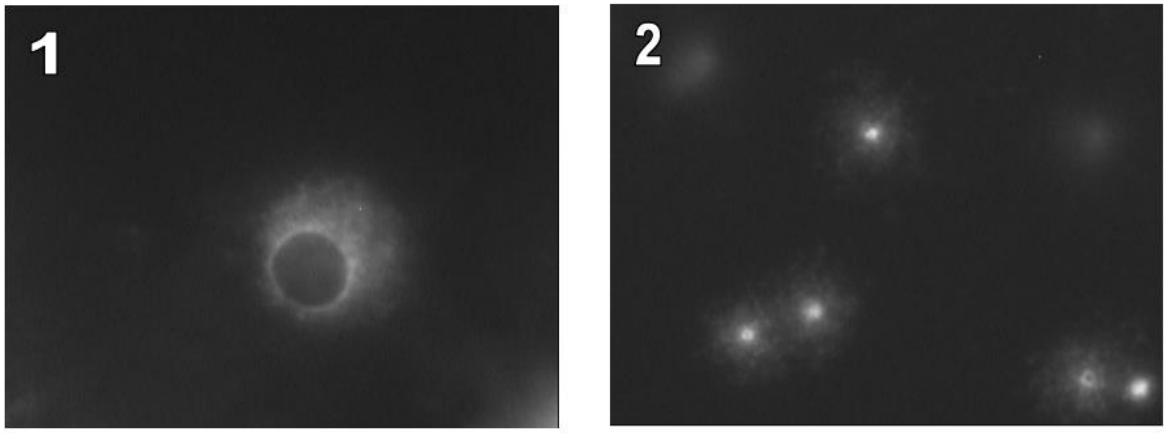


Figure 4.8: *Comet assay #5; S. cerevisiae control cells + 10 mM of hydrogen peroxide (1) and cells exposed to 25 mM of hydrogen peroxide + 10 mM of hydrogen peroxide (2) (amplification 1000x)*

YCA-Trial 6. Since the fifth assay showed no formation of comet tails, but the cell DNA was exposed, this sixth assay was performed to verify if the percentage of agarose used in the gel and the voltage used in the electrophoresis run had any significant influence on the formation of the comet assay tail.

The Figure 4.9 represents control conditions with a 2.5% gel percentage and an electrophoresis run of 28 V (1) and a 1.5% gel percentage with an electrophoresis run of 32 V (2), 10 mM of H_2O_2 positive control cells with a 2.5% gel and an electrophoresis run of 28 V (3) and a 1.5% gel percentage and an electrophoresis run of 32 V (4), 25 mM of H_2O_2 positive control cells with a 2.5% gel percentage and an electrophoresis run of 28 V (5) and a 1.5% gel percentage with an electrophoresis run of 32 V (6). Succinctly, cells were exposed to different electrophoresis run voltages and to different percentage of agarose to perform the assay. These changes did not show any significant difference in obtaining comets, which means that agarose gel percentage and voltage allegedly did not have a direct influence in obtaining comet tails, or perhaps these were still not the adequate parameters. The voltage parameter was set to 28 V and 32 V, because it will change the amperage, causing the electric field to flow with more or less intensity. This change would be expected to cause a higher or lower DNA flow in the gel matrix.

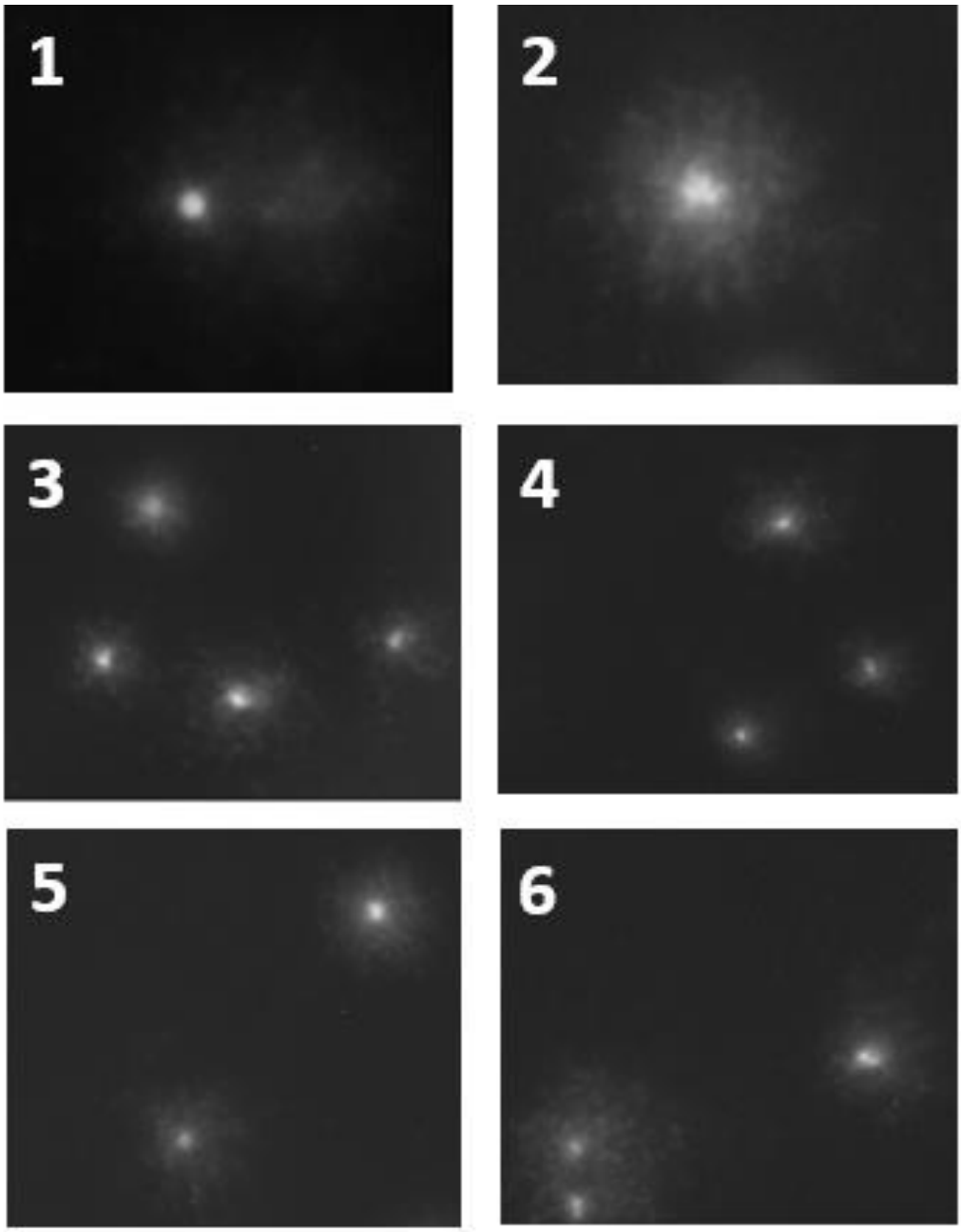


Figure 4.9: *Comet assay #6; S. cerevisiae control cells exposed to a 2.5% gel percentage and an electrophoresis run of 28 V (1) and a 1.5% gel percentage with an electrophoresis run of 32 V (2); S. cerevisiae cells exposed to 10 mM of H2O2 with a 2.5% gel and an electrophoresis run of 28 V (3) and a 1.5% gel percentage and an electrophoresis run of 32 V (4); S. cerevisiae cells exposed to 25 mM of H_2O_2 with a 2.5% gel percentage and an electrophoresis run of 28 V (5) and a 1.5% gel percentage with an electrophoresis run of 32 V (6) (amplification 1000x)*

YCA-Trial 7. Since the problem to obtain comet tails persisted, a seventh assay was performed to confirm if the electrophoresis running conditions and the percentage of agarose used in the gel did not have a direct influence. In this assay the gel was made with 1.5% and 2.5% of low melting agarose. The cells were exposed to a positive control of 25 mM of H_2O_2, all the samples variations were submitted to a 10, 20 and 30 min electrophoresis run at 32 V.

The Figure 4.10 represents control cells that were exposed to a single gel agarose with a concentration of 1.5% and a 10 min electrophoresis run (1) control cells that were exposed to single gel

with 2.5% of low melting agarose and a 30 min electrophoresis run (2) cells that were exposed to 25 mM of H_2O_2 with a single gel percentage of 1.5 and an electrophoresis run of 10 min (3) and cells that were exposed to 25 mM of H_2O_2 with a 2.5% of single gel and an electrophoresis run of 20 min. There was no significant difference in the results of all the conditions tested. A comet tail pattern could not be obtained, which most likely means that the percentage of agarose and the duration of the electrophoresis run did not have a direct influence in obtaining comet tails, although further tests must be made to confirm it.

In order to confirm the obtained results from all the comet assay tested conditions, the assays were made in duplicated, however no significant changes were observed. The duplicated results were not showed in the thesis.

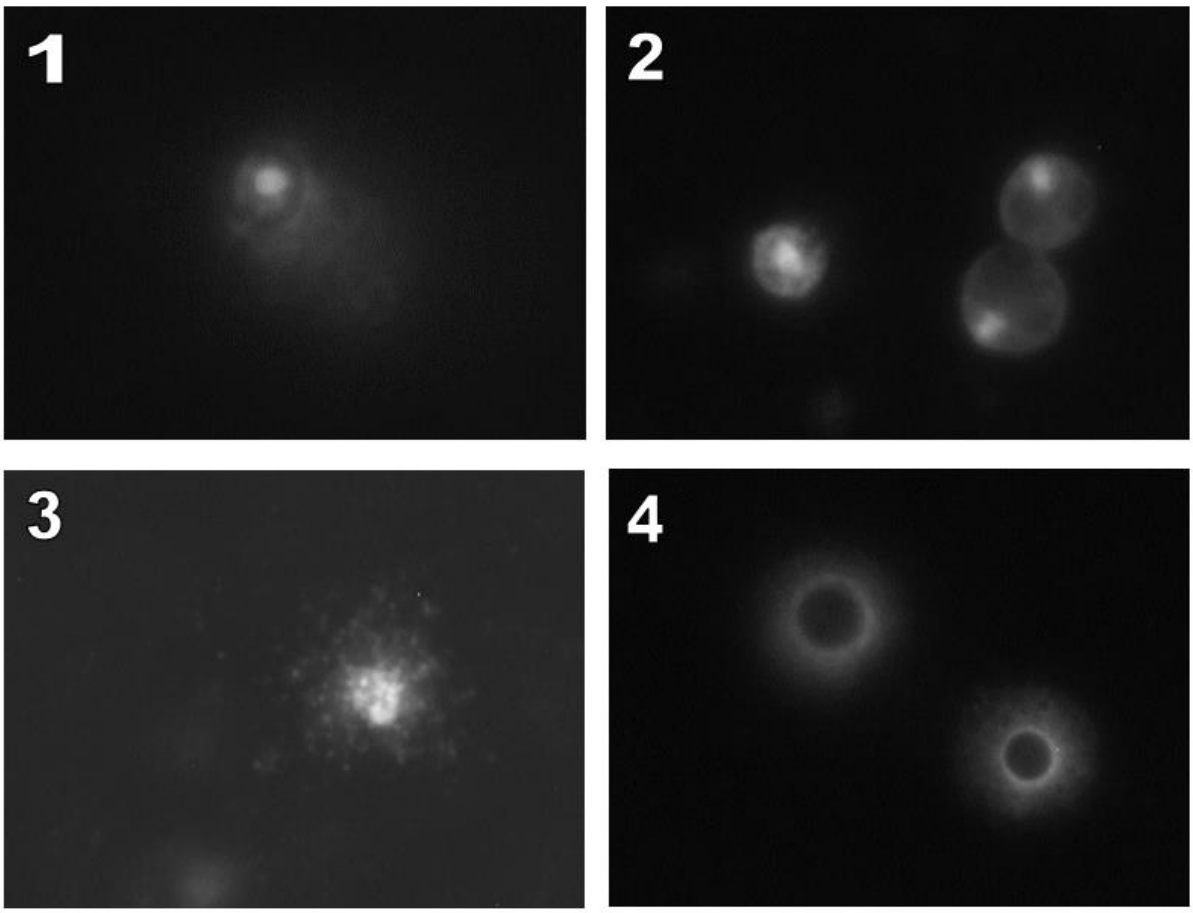


Figure 4.10: *Comet assay #7; S. cerevisiae control cells exposed to a 1.5% gel percentage and an electrophoresis run of 10 min (1) and a 2.5% gel percentage with an electrophoresis run of 30 min (2); S. cerevisiae cells exposed to 25 mM of hydrogen peroxide with a 1.5% gel percentage and an electrophoresis run of 10 min (3) and a 2.5% gel percentage with an electrophoresis run of 20 min (4) (amplification 1000x)*

4.2.2 Evaluations of *S. cerevisiae* strains

After having performed several comet assays with no reproducible consistent results, it was decided to clarify if the strain used in comet assays, *S. cerevisiae* VL3, had some problems related to cells wall degradation, that could make the strain not suitable for the comet assay. To solve this doubt the comet assay was preformed with three diferent comercial strains of *S.cerevisiae*: VL3, VR5 and

L331, with a negative control condition and positive control of 50 mM of H_2O_2, a concentration quite damaging to the cells. The lysis buffer 2 and the electrophoresis buffer 3, were chosen to perform this assay.

The Figure 4.11 represents negative control cells of *S. cerevisiae* VL3 (1) and positive control of the same cells (2), a negative control of *S. cerevisiae* VR5 cells (3) and the positive control (4) and a negative control of *S. cerevisiae* L331 cells (5) and the positive control (6). After these tests it was confirmed that the doubt raised was not valid since the results showed that there were no significant changes in cell degradation or tail appearance. The problem does not seem to be related to the strains tested but it should be linked to one or several factors related to the experimental procedure followed for the comet assay.

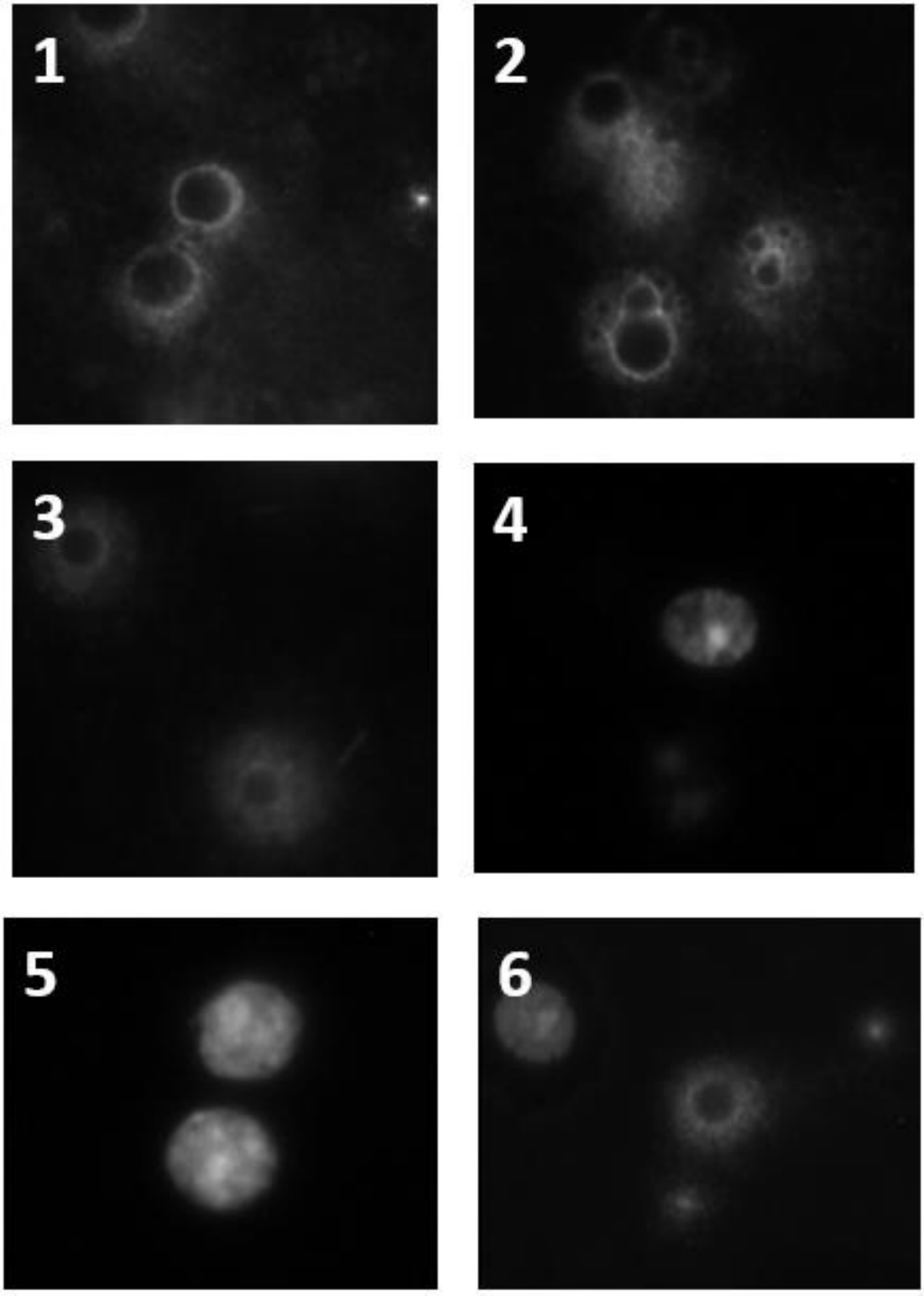


Figure 4.11: *Comet assay: S. cerevisiae VL3 stain control cells (1) and VL3 cells exposed to 50 mM of hydrogen peroxide (2); S. cerevisiae VR5 control cells (3) and VR5 cells exposed to 50 mM of hydrogen peroxide (4); S. cerevisiae L331 control cells (5) and L331 cells exposed to 50 mM of hydrogen peroxide (6) (amplification 1000x)*

4.3 Alterations of genes expression levels

4.3.1 Evaluation of RT-qPCR parameters

The RT-qPCR conditions were optimized for all the target genes: *Apn1*, *Apn2*, *Rad27*, *Ntg1*, *Ntg2* and *Cdc55* and the reference genes, *Taf10* and *Alg9*, by confirming that reactions had a linear regression coefficient (R^2) above 0.95% and the efficiency value was between 88% and 100%, as it summarized in Table 4.1.

Table 4.1: *Threshold RT-qPCR reaction parameters of target and reference genes*

Gene		Annealing temperature (ºC)	Efficiency (%)	Slope	Y intercept	R^2	Reference
BER genes	*Apn1*	52	98	-3.361	24.083	0.997	This study
	Apn2	52	100	-3.314	26.196	0.992	This study
	Rad27	52	96	-3.416	24.524	0.998	This study
	Ntg1	48	88	-3.649	25.863	0.993	Valério et al., 2016
	Ntg2	48	94	-3.484	21.605	0.993	Valério et al., 2016
PP2A	*Cdc55*	49	97	-3.396	21.985	0.997	Valério et al., 2016
Reference genes	*Alg9*	58	97	-3.398	23.906	0.989	Teste et al, 2009
	Taf10	58	92	-3.536	23.103	0.996	Teste et al, 2009

4.3.2 Relative gene expression evaluation in *Saccharomyces cerevisiae* exposed to different MC-LR concentrations

4.3.2.1 BER genes

S. cerevisiae cells were exposed to different concentrations of microcystin-LR: 1 nM, 10 nM, 100 nM and 1 µM and subsequently the differences in the expression levels of BER genes (*Apn1*, *Apn2*, *Rad27*, *Ntg1* and *Ntg2*) and *Cdc55* gene relative expression was evaluated by Real-Time RT-qPCR (detailed protocol described in the annex VII).

Six biological replicates were made in order to obtain more consistent results. However, since reference genes *Taf10* and *Alg9* presented considerable variations in some of them, that would cause many errors and it would not be possible to observe a tendency among the results, it was decided to present and discuss only the assays that did not presented variations in the reference genes. A normalization was applied to the results presented.

In Figure 4.12, it can be seen the *Apn1* gene relative expression, considering that control is the baseline 100%, the expression values vary between 75% and 119% when *S. cerevisiae* cells were exposed to different microcystin concentration (1 nM, 10 nM, 100 nM and 1 µM). For the two lower doses of MC-LR tested (1 nM and 10 nM), there is a tendency for the underexpression of the gene. On the contrary, for the higher doses (100 nM and 1 µM), there is an overexpression of Apn1 gene.

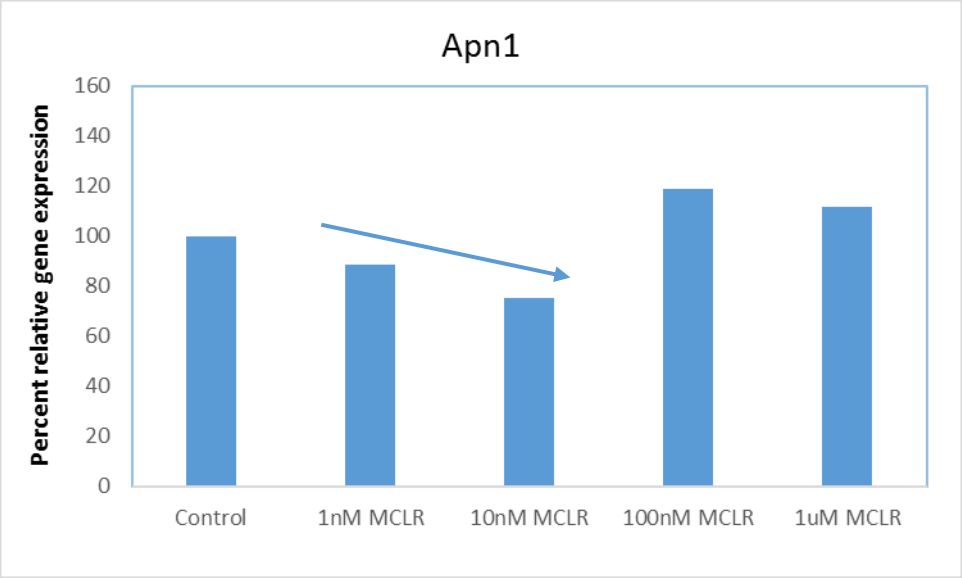


Figure 4.12: *Apn1 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of three biological replicas were used to obtain these results)*

The Figure 4.13, represents *Apn2* gene relative expression. Gene relative expression values varied between 100% and 70%, among all microcystin concentrations. Overall there is a tendency for the gene underexpression when compared with the control, and a correlation could be assessed between lower MC-LR and lower relative gene expression. The highest MC-LR concentration (1 µM) is also associated with the highest relative expression, which is equal to the control.

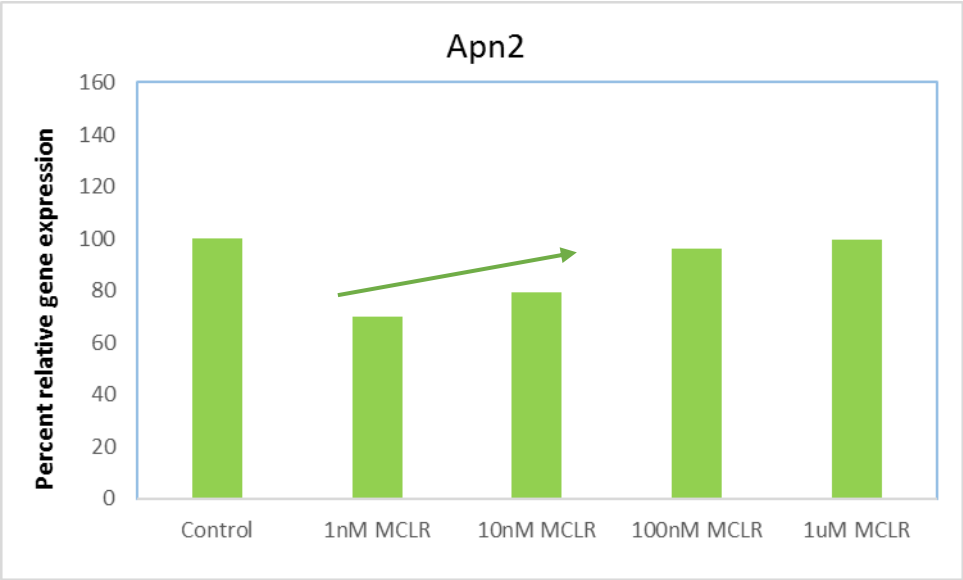


Figure 4.13: *Apn2 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of three biological replicas were used to obtain these results)*

For the *Rad27* gene relative expression values vary between 99% and 142%, as is represented in Figure 4.14. When compared to the control, there seems to be a dose-response, since there is a tendency for gene overexpression with increasing MC-LR concentration, except for the lowest MC-LR concentration (1 nM) which is slightly underexpressed.

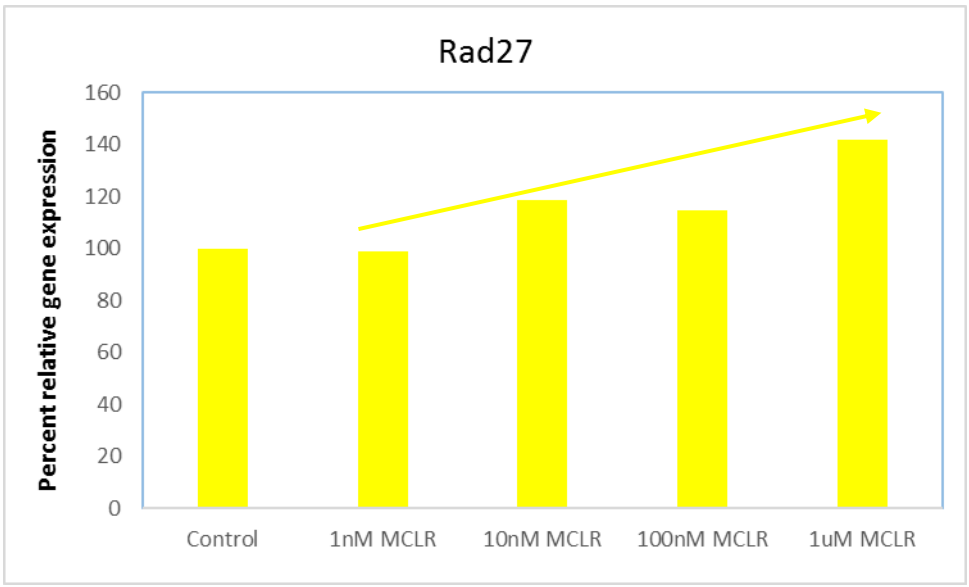


Figure 4.14: *Rad27 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of three biological replicas were used to obtain these results)*

The Figure 4.15, represents *Ntg1* gene relative expression, with values that vary between 100% and 75%. All concentrations of MC-LR show a repression of relative gene expression when compared with the control. There is a tendency which is similar to the *Cdc55* gene, among the lowest MC-LR

concentrations (1 nM and 10 nM), the 10 nM display a higher repression effect. The sample exposed to 100 nM MC-LR has the highest relative gene expression, but is still underexpressed when compared with the control.

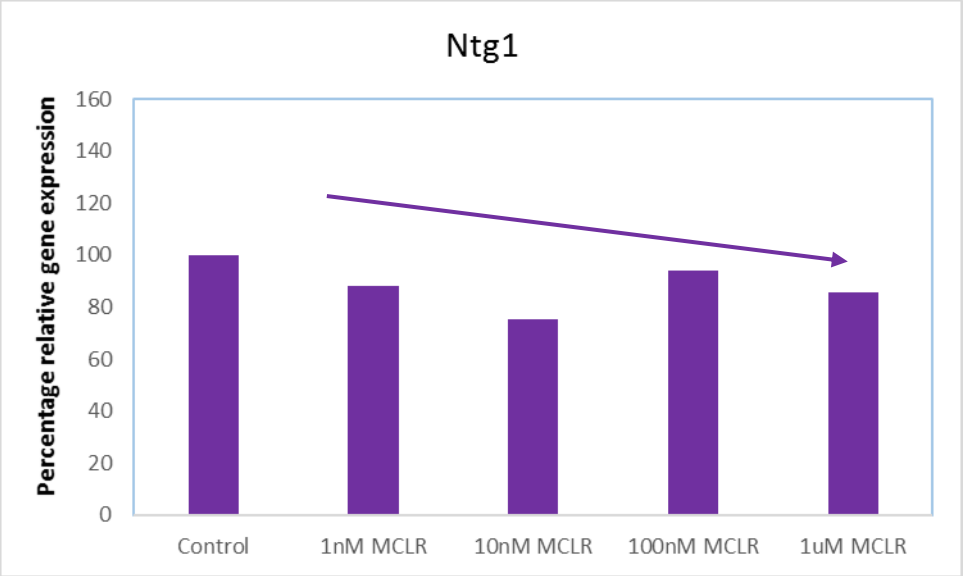


Figure 4.15: *Ntg1 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of three biological replicas were used to obtain these results)*

The *Ntg2* relative gene expression, has values that vary between 100% and 81%, as represented in Figure 4.16. *Ntg2* differs from all the other gene mentioned above, since it shows a clear dose-response tendency, where the higher MC-LR concentrations are associated with lower relative gene expression.

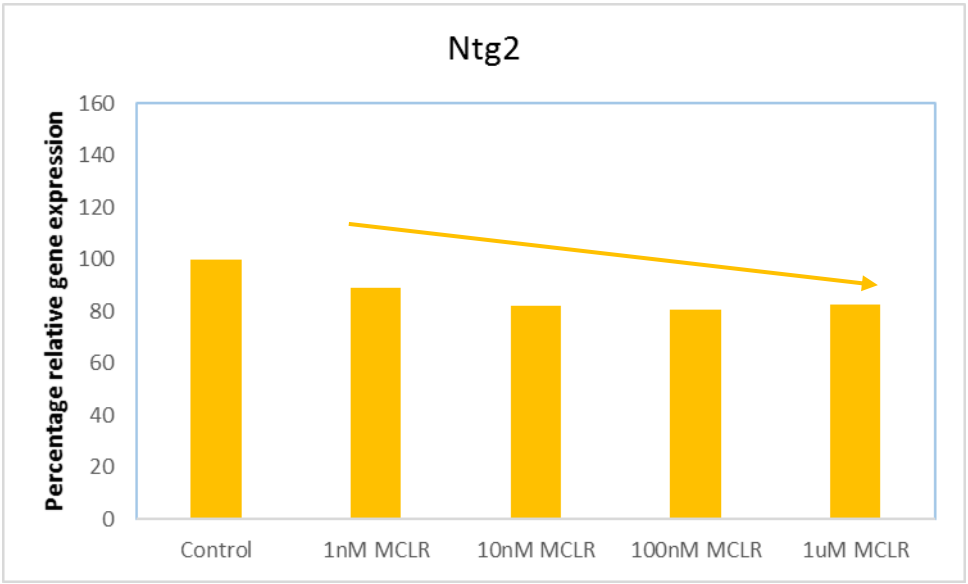


Figure 4.16: *Ntg2 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of three biological replicas were used to obtain these results)*

4.3.2.2 Protein phosphatase gene

In Figure 4.17, it can be seen the *Cdc55* gene relative expression values that vary between 78% and 49%, when exposing the yeast cells to different microcystin concentrations. Overall all samples exposed to MC-LR concentrations show an inhibition in the expression of *Cdc55* gene when compared to the control (without MC-LR), among the two lowest MC-LR concentrations (1 nM and 10 nM), it is observed a more pronounced effect with 10 nM. The highest concentrations tested (100 nM and 1 µM) show a similar tendency.

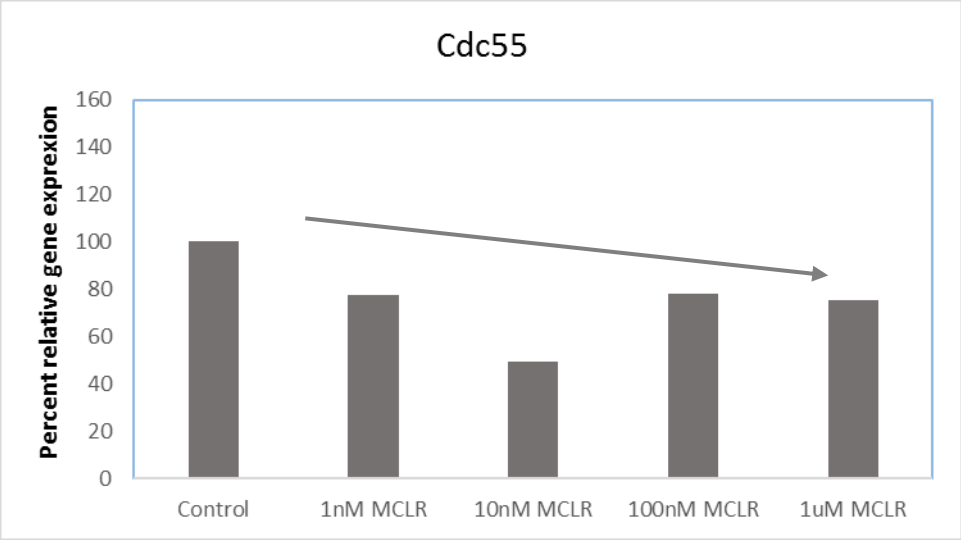


Figure 4.17: *Cdc55 relative gene expression (%) when yeast cells are exposed to 0 nM (control), 1 nM, 10 nM, 100 nM, 1 uM of MC-LR (the average of four biological replicas were used to obtain these results)*

5. Discussion

5.1 *Saccharomyces cerevisiae* viability when exposed to different concentrations of MC-LR

The MTT assay is a method to determinate cell viability/ cell density, through the reduction of 3-(4,5-Dimethyl-2-thizaolyl)-2,5-diphenyl-2H-tetrazolium bromide into a colored product, formazan, (by mitochondria dehydrogenases), to which the cell membrane is impermeable (Dias et al., 2009).

Previous studies, such as Chong et al. (2000) and Dias et al. (2009), show that the assay is well established for microcystin, however these studies were applied to mammal cell lines, which do not have cell wall, and where cultures are in an adherent subtract. These studies reveal that there is a negative correlation between cell viability and MC-LR concentration, where there is a significant dose/time dependent cytotoxic effect of MC-LR on cell viability (Chong et al., 2000; Dias et al., 2009).

To test if the assay was working correctly with the model organism used in this work, *S. cerevisiae*, two positive controls were used, which were previously known to cause serious damage to cells, including yeast cells (Valério et al., 2014), and would consequently affect cell viability by reducing the number of viable cells (Dias et al., 2009). Both positive controls, SDS (a detergent) and H_2O_2 (an oxidizing agent), showed a significant decrease (dose dependent) in cell viability. These two positive controls allowed the confirmation that the MTT assay, regarding *S. cerevisiae* cytotoxicity, was applicable. However, when using microcystin as chemical component, the results were not so reproducible and concise.

There are not many studies that use the MTT method with yeast cells, so it was difficult to have a reference for this study. Kiruthika & Padma (2013), used the MTT method in *S. cerevisiae*, however their method was not similar to the one used in this thesis, namely the timings and the reagents used were different. The fact that the MC-LR showed not to be cytotoxic for *S. cerevisiae* might be related to the fact that the absorbance readings were very low, which means that the incubation time for the MTT reduction, or, the incubation time for the formazan dye to solubilize might be too short (MTT Cell Proliferation Assay, 2017 from https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx, accessed on January 2017).

Another possible reason for the difficulties of applying the MTT test in yeast cells exposed to MC-LR may be due to the microcystin molecular size. Since microcystins are molecules with relatively high molecular mass (900-1100 Da) (Campos et al., 2016) it may have variable entrance amounts during the MTT assays. This hypothesis could be confirmed by electron microscopy or other microscopic method with increased resolution and the use of directed antibodies; however, the installations used did not have this methodology available at the moment. However, regarding the results obtained by RT-qPCR, the MC-LR apparently can enter the cells.

Another possible reason for the assay not be working properly may be related to the lack of cell synchronization. Since the cells used are in a suspension culture they may, and probably are, in different life stages, different development levels, which may influence the percentage of MC-LR entrance into the cell and consequently the percentage of MTT reduction. Although a protocol for cell synchronization, similar to the one described in Chong et al. (2000) was used in this work to test the theory (results not showed), the MTT assay still did not produced reliable results.

The MTT assay was previously suggested to be a sensitive and reliable method for measuring cell viability, however based on the tests performed in this thesis, it seems that this does not apply when evaluating microcystins cytotoxicity in yeast cells. Furthermore, among other toxic characteristics, microcystin-LR is a chemical compound responsible for inducing the formation of ROS (reactive oxygen species) which triggers oxidative stress in cells that will lead to a decrease in NADPH, which means lower cell viability, and therefore a low absorption intensity when extracted with DMSO. Because of this, it can be assumed that NADPH may provide a direct indicator of oxidative stress (Petrat et al., 2003). The ROS formation could also interfere / influence the results of the MTT assay. Regarding the problems when exposing the cells to MC-LR when performing the MTT assay, a trend can be seen towards higher cell viability when exposed to higher concentration of microcystin.

Apparently, MC-LR is not cytotoxic for *Saccharomyces cerevisiae*, although these results should be confirmed with other methods that access cell viability (e.g. flow cytometry or Neutral Red).

Due to all these adversities, some authors recommend that cell viability based on MTT reduction be asserted by cell counting under a microscope (Li et al., 2007), although it is a more time-consuming method, it would provide an idea of the interaction among the MTT and the cell.

5.2 *S. cerevisiae* genotoxicity tests

5.2.1 Comet assay

The comet assay is a method used to measure DNA damage in individual cells.

Despite its great potential, the YCA has been applied only on a few species including *S. cerevisiae*, *S. pombe* and *Candida* sp. (Staneva et al., 2013). Some drawbacks make it a difficult to obtain reliable results, such as the lower DNA content of yeast cells and the need for cell wall digestion prior to electrophoresis (Oliveira et al., 2012).

While trying to optimize the assay, some experimental conditions were changed, including the composition of the lysis buffer and the lysis duration, the electrophoresis duration and power voltage, the agarose gel concentration and the solutions involved in the process. After some unsuccessful trials, there was some progress since we could observe the digestion of the cell wall and membrane, thus exposing the cell's DNA, which is critical to allow its migration under the electric field actions. However, no comet tails were observed following yeast cells exposure in the positive controls, despite of the conditions tested. Because of this, the comet assays were not executed in cells exposed to MC-LR.

Hydrogen peroxide is an oxidizing agent and a mutagen known for inducing DNA breaks. These were the reasons for it being chosen as the positive control for YCA (Staneva et al., 2013).

Miloshev et al. (2002), were the first researchers that described the application of the YCA, using *S.cerevisiae* as a model. This work showed that to overcome the problem of the yeast cell wall, which prevented DNA from migrating out of the nucleus when applying the electric field, cells should be embedded in agarose and the cell wall should be disintegrated with hydrolytic enzymes, to obtain intact spheroplasts that would form the comet tails (Miloshev et al., 2002).

In the first test the YCA failed in obtaining comets, the cell wall was still intact, which did not allow the migration of DNA fragments. The fact that the enzyme lyticase only acted for 5 min, may have not been sufficient time, to obtain intact spheroplasts.

In the second assay the YCA failed in obtaining comets, the cells membrane or wall looked intact, which did not allow the migration of DNA fragments. Possibly the exposure times and conditions were still not optimized. Another possible explanation might be the fact that a small fraction of cells could be resistant to cell wall enzymatic degradation, and thus the cell wall was not degraded even after being treated. Resistant cells are easily distinguishable by their well-defined borders and intense homogeneous staining (Oliveira & Johansson 2012).

In the third test the YCA showed some promising results, the enzymatic degradation worked, on most of the cells, when lyticase acted for 30 min in a control environment with a temperature of approximately 30ºC. The DNA is exposed but no comets are visible after the YCA. One of the reasons for not obtaining clear and good comets, when compared with mammalian cell comets, is that *S. cerevisiae* chromatin is much less compact and heterochromatic domains are rare in the nucleus. The haploid yeast cell nucleus contains approximately 13 Mbp in comparison with human cell nucleus which contains about 3300 Mbp (Rank et al., 2009). Therefore, the smaller and partitioned DNA fragments migrate faster, making a distorted comet tail, when compared with mammal cells (Miloshev et al., 2002). In this assay cells were treated with 10 mM, 25 mM and 50 mM of H_2O_2, in order to see different DNA damage degrees. In Azevedo et al. (2010), a dose-response relationship was found between H_2O_2 concentration and tail length until 10 mM. However, when cells were treated with 50 mM H_2O_2, tail length did not increase when compared with cells treated with 10 mM of H_2O_2, this might indicate a limited capacity of the genomic DNA to unwind and migrate in an electric field (Azevedo et al., 2010).

After not observing DNA migration, we came with the hypothesis that the problem might have been associated with electrophoresis conditions. In the fourth test the YCA was performed using another electrophoresis buffer (2) where 1 M of NaCl was added, to decrease the conductivity, or ionic strength of the buffer, slowing down the migration of all DNA fragments (Stellwagen & Stellwagen, 2002). If the migration rate is high, DNA fragments could migrate out of the gel matrix, preventing comet formation (Stellwagen & Stellwagen, 2002). However, the assay was not successful since the cell wall remained intact. Something must have occurred with enzymatic degradation, since it was not successful. One of the most difficult obstacles is cell-wall degradation is choosing the proper enzyme and the right protocol. Most YCA studies use 2 mg/mL of zymolase 20 T (Miloshev et al. 2002; Peycheva et al. 2009; Rank et al., 2009; Azevedo et al. 2011; Oliveira et al., 2012). One of the problems with the conditions tested might be the fact that zymolyase is much more effective when forming yeast protoplasts than lyticase, which implies that the exposure time and lyticase concentration applied to the study should have been higher. Nerveless, some YCA studies, (e.g. Rank et al., 2009), had some difficulty with cell wall degradation while applying zymolase 20 T, and had to increase it to 100 T, which has a five-fold higher enzyme activity (Rank et al., 2009). One possible explanation for the differences in successfully breaking down yeast cell wall, might be different enzyme sensitivities between different yeast strains (Rank et al., 2009).

Since the previously assay showed that there was no destruction of cell wall or membrane, a new lysis buffer was tested in the following assay, fifth test. The new lysis buffer showed some promising results, since the enzymatic degradation worked on most cells, and the DNA was exposed. The new lysis buffer (2) differed from the first since it was added Triton-X-100 (1% v/v), instead of N-

Lauroylsarcosine, and the pH was adjusted to 12, to become more alkaline. The same buffer was used in Miloshev et al. (2002) and Peycheva et al. (2009), although the pH level used in the previous assay was 10. Triton-X-100 is a detergent with a protein denaturant potency, so it has been used on YCA due to its potential for lysing protoplasts (Sigma Aldrich, 2017 from http://www.sigmaaldrich.com/catalog/product/sigma/l5125?lang=pt&region=PT, accessed on March 2017). Since the cells exposed to different concentrations of H_2O_2 show a significant difference in cell degradation, it was assumed that the new alkaline lysis buffer was more adequate for the assay. Various studies show that alkaline or neutral variant of the CA can produce distinct results, therefore pH is a determinant factor in the assays. One of the main difference is that the alkaline assay is much more sensitive to DNA damage detection, since it reveals double and single stranded DNA breaks and alkali-labile sites, however there is an increased difficulty in choosing the best variant, when applied to yeast, since the assay sensitivity greatly depends on the compounds studied (Peycheva et al., 2009). Besides the difficulty in knowing which variant is the most appropriate, when comparing with comets of higher-eukaryotic cells the alkaline version of YCA produces more heterogeneous results, making it more difficult to interpret (Peycheva et al., 2009).

After having the DNA exposed but no migration to account for, the goal was to observe a distinct direction of DNA migration. To achieve that goal two low melting agarose percentages were used in the single gel assay, the usual 1.5% and a 2.5%, corresponding to a final low melting agarose concentration of 0.7% and 1.3%, respectively, since the gel mix is made with 50% of cells suspension and 50% of agarose. Two voltages were also used in this assay, the usual 28 V and another of 32 V, for the electrophoresis run of 10 min (sixth test). The increase of low-melting agarose concentration, could provide a more stable electrophoresis matrix for damaged DNA migration, from cells with low chromatin content, which is the yeast case (Azevedo et al., 2010). This increase would technically prevent the excessive migration of small DNA fragments that could eventually run out of the electrophoresis matrix, which would be impossible to access and identify. The voltage is the parameter that determinates DNA movement, it is the voltage gradient over the gel that will be responsible for DNA migration. The voltage gradient depends on resistance, so it will be higher when in contact with gel surface. Increasing the amount of buffer in the tank, will increase the current and consequently reduce the resistance (Comet assay, 2017 from http://cometassay.com/index_files/Page345.htm, accessed on March 2017). A new voltage was used to increase the current and try to obtain DNA migration by reducing the resistance when in contact with the gel surface. However, cells that were exposed to different electrophoresis and different agarose percentage, did not show any significant difference regarding comet formation. It seemed that agarose gel percentage and voltage did not had a direct influence in comet formation, or perhaps the conditions were still not ideal.

Despite all protocol alterations that were introduced, there was still no DNA migration, so in the next assay, (seventh test), there was an attempted to extend the electrophoresis running time, for 10, 20 and 30 min. The two percentages of agarose were still tested to confirm eventual influence on DNA migration. The percentage of damaged DNA on comet tail, when cells are previously treated with H_2O_2, is strongly influenced by the electrophoresis time, some authors suggest it can range up to 40 min, and voltage gradient can rise up to 1.6 V/cm (Azqueta et al., 2011). The cells exposed to different electrophoresis times and percentage of agarose did not show any significant difference and, a pattern could not be obtained.

Even after all YCA variables tested, the results were not conclusive and comets tails could not be obtained from *S. cerevisiae* cells. Some possible reasons are described above, however, other conditions/situations may have prevented comet formation.

Sometimes no difference could be observed between the incubation with various concentrations of H_2O_2 and the negative control. In Azevedo et al. (2010), there is a protocol optimization to overcome this situation, which increases the low-melting agarose concentration, decreases detergent concentration and decreases the pH of the lysis and electrophoresis buffers (Azevedo et al., 2010). However, this suggestion to use a neutral version of the YCA, is the opposite of what most studies propose. In summary, there is little concordance among authors, regarding which are the right conditions and protocol to apply to yeast cells.

Another problem may arise from the fact that cells at different life stages produce variable comets. When cell cultures proceed towards stationary phase, there is an increase of the comet tail length, however, cells in stationary phase do not show comet features, possibly due to resistance to spheroplasting enzymes, such as zymolyase or liticase. Yeast cells do not spheroplast well at higher OD, therefore, cells in stationary phase have an increased resistance to cell wall-degrading enzymes (Azevedo et al., 2010). The culture OD used was the same for each assay, ca. OD 0.1 (Valério et al. 2014), which ensures that it is not in the stationary phase. Since cell life stages were not controlled, this could be a parameter that would require more control and attention in future YCA.

The comet assay detects single-strand breaks and double-strand breaks, however some chemicals could damage DNA bases by oxidation, which will leave the sugar phosphate backbone intact. Since these damages will not lead to DNA breaks they would not be detected by the comet assay (Oliveira & Johansson 2012). If the damages induced by the chemicals tested in this study do not provoke DNA breaks, there would be no comet tail formation, which could be another explanation for the absence of comets.

If damages consist mostly of DNA double-strand breaks, there is an increase in electrophoretic mobility when comparing with single-strand breaks, this may be a reason for sometimes not seeing DNA fragments on the photo, since they all migrate out of the electrophoresis matrix (Oliveira & Johansson 2012).

The fact that YCA uses lower eukaryotes, such as *S. cerevisiae*, that have low cellular DNA content, will cause irregular comet tail shapes and increase the difficulty in the implementation of quantification systems (Oliveira & Johansson 2012). Since one of the problems was the insufficient amount of DNA in yeast cells, Rank et al. (2009) tried using tetraploid *S. cerevisiae*. However, the amount of DNA in the nuclei was still too low for a proper microscopic analysis. Rank et al. (2009) recommends that tetraploid *S. cerevisiae* yeast cells should not be developed further in the comet assay.

Sometimes some of the images acquired, showed that yeast DNA formed images resembling halos, rather than comets tails (e.g. test seven) even when there was sufficient damage to produce tails. This phenomenon could not been explained and it requires further investigation (Comet assay, 2017 from http://cometassay.com/index_files/Page345.htm, accessed on March 2017.) Even the YCA protocols that produced some results such as Miloshev et al. (2002) Peycheva et al. (2009), Azevedo et al. (2010), Oliveira et al. (2012), among others, may present some blobs and granules in the tails, which most probably represent fragments of yeast chromatin, that reflects the weak structure of yeast nucleus (Peycheva et al., 2009). Some of the comets images acquired in these studies were relatively weak. This allows to conclude that even if the YCA produces acceptable results these will never be as clear as the traditional assay (CA) and sometimes these results are handled too lightly and should not be accepted in an irrefutable way.

When contacting some field specialists about these difficulties, their response was that it was very hard to obtain comets in *Saccharomyces* spp. cells, the results were not always achieved and only after a year trying did they had some results to show for.

The attempts to obtain solid results with YCA were interrupted due to several difficulties both at a financial and availability level. Each YCA takes about 5 days to complete, including photograph acquirement, in addition the microscope used was not always available, so it was difficult to schedule the assays. Over the thesis project there were also some problems with the equipment, namely the microscope used to visualize the cells, becoming unusable, which delayed the project and made it more difficult to conclude the YCA optimization process.

The assay conditions may not have been the best and some reagents should also have been tested to make the results more consistent, (e.g. test the enzyme zymolase to disintegrate cell wall). However, it was not possible, mainly because it was difficult and expensive to order reagents in such a short notice.

5.2.2 *Saccharomyces* spp. genotoxicity when exposed to different concentrations of hydrogen peroxide

Different strains of yeast may have different sensibility to cell wall digesting, so there was a need to verify whether different *S. cerevisiae* strains would react differently to the same YCA conditions, being more easy or difficult to form comet tails (e.g. *K.lactis* strain is about six times more sensitive to Zymolyase when compared to *S. cerevisiae* (Staneva et al., 2013). The concentration of H_2O_2, when applied to different strains, may also influence the DNA damage extent, for example 25 µM of hydrogen peroxide has minimal effect while inducing DNA damage in *K. lactis*, however in *S. cerevisiae* it causes great damages (Staneva et al., 2013).

The strains tested in this work were *S. cerevisiae* VL3, VR5 and L331 (commercially available), each of them was exposed to a positive control of 50 mM of hydrogen peroxide and compared to the negative control. There were no significant changes in cell degradation or tail appearance, which means that the problem was not linked to the strain, but it is most likely related with YCA parameters.

5.2.3 Relative expression levels of BER genes

The Real-Time qPCR assay provides the relative gene expression, which is the variation of gene expression between two samples, the studied condition and the control condition. The results obtained by RT-qPCR were asserted using both reference genes *Alg9* and *Taf10*.

In theory MC-LR may cause deleterious effects on DNA which will lead to an increased expression of BER genes, when trying to repair the damages. The overexpression of these genes suggests that they are trying to overcome the effects of the toxin, and it is expected to occur when the toxin concentration is low, because it causes few DNA damages. On the contrary underexpression/repression of these genes suggests that the DNA had extended deleterious effects that could not be overcome by the repair mechanism, and it should be more evident when the toxin concentration is high.

Regarding the BER genes relative expression, it was found that *Apn1*, Table 5.1, showed a tendency for being underexpressed regarding the lowest concentrations (1 nM and 10 nM) and overexpressed in the highest concentration of MC-LR (100 nM and 1 µM). These results were surprising since it is the opposite of what was expected. Furthermore, the concentration at which the relative gene expression was the highest was 100 nM.

Apn2 relative gene expression, Table 5.1, shows that genes exposed to every MC-LR concentration were underexpressed when compared with the control (without MC-LR). A dose-response tendency could be seen, genes exposed to higher MC-LR concentration showed a tendency to be more relative expressed, but never exceeding the control relative expression. This might suggest that higher DNA damages would cause an increased gene expression to try to overcome the damages. However, that did not fully happen, since the *Apn2* gene expression was still lower than the control.

Rad27, Table 5.1, showed a slight relative gene overexpression in the cells exposed to different MC-LR concentrations, when compared with the control. A dose-response tendency could be seen, since genes exposed to higher MC-LR concentration show a tendency to be more expressed, except for the fact that the highest relative gene expression was at 10 nM of MC-LR. These results might suggest that higher DNA damages would cause an increase in relative gene expression to try to reverse them.

A previous study compares yeast life span with BER activity (Maclean et al., 2003). It shows that losses of enzymes Ntg1p and Ntg2p increases both spontaneous and hydrogen peroxide induced mutation frequencies, though it generally does not cause cell death. A single deletion of these enzymes has little or no effect regarding life span, however combined deletions exhibit a decreased cell survival (Maclean et al., 2003).

Regarding the AP endonucleases, Apn1p is the most important. Without it the spontaneous mutation rate would increase a lot, becoming more sensitive to hydrogen peroxide. The Apn2p may be conferring resistance to ROS induced damages in an alternative pathway to the Apn1p. Similar to the BER DNA glycosylase, the combined loss of both endonucleases exhibited extremely poor cell survival. The AP endonuclease overexpression, Apn1p and Apn2p, could restore a full chronological life span to yeast cells (Maclean et al., 2003).

No further studies were found using the genes, *Apn1*, *Apn2* and *Rad27*, particularly with cells exposed to microcystin, thus it is difficult to make comparisons.

Apn1 and *Apn2* genes are members of the endonuclease IV and are part of the BER system. *Apn1* encodes a protein APN1, which will later be facilitated from either the nucleus or cytoplasm, into the mitochondria to repair damaged DNA (Morris et al., 2012). *Apn2* major function is to remove abasic sites from DNA (Fraser et al., 2003) and *Rad27* is a flap endonuclease, which takes part of the BER system and is involved in DNA repair mechanisms (Ohnishi et al., 2004). Theoretically, it would be expected to see the gene overexpression with the lowest MC-LR concentrations and an underexpression with the highest.

The BER gene, *Ntg1*, shows a tendency for being underexpressed in all concentrations when compared with the control, Table 5.1. Conversely, in Valério et al. (2016a), the lower concentration of MC-LR (1 nM) induced an overexpression of *Ntg1* gene in opposition to the underexpression seen in the presence of 100 nM and 1 µM MC-LR (Valério et al., 2016a).

The gene *Ntg2*, shows a tendency for being underexpressed at all MC-LR concentrations. A dose-response could be seen regarding higher MC-LR concentrations associated with lower relative gene expression, Table 5.1. This could be explained by the fact that higher concentrations of MC-LR cause higher DNA damages that could not be overcome by this gene overexpression. However, in Valério et al., (2016a), the *Ntg2* gene in the presence of 1 nM and 100 nM of MC-LR, had an overexpression in contrast with what happen whit the highest concentration of MC-LR (1 µM) which suffered and underexpression of the gene (Valério et al., 2016a).

The *Ntg1* and *Ntg2* are genes from BER system which eliminates small DNA lesions that could be caused by exposure to environmental agents or ROS (known mechanisms of MC-LR toxicity) among others. In Valério et al. (2016a), the *Ntg1* and *Ntg2* expression levels obtained suggest that higher MC-LR concentrations caused toxic effects mainly in the mitochondria. The study also suggests that the differences between genes (*Ntg1* and *Ntg2*) could be attributed to the protein's action location, since *Ntg1* acts both in the nucleus and mitochondria and *Ntg2* only acts in the nucleus. The results were latter supported from the fact that higher ROS levels and early apoptotic response were detected in yeast cells (Valério et al., 2014; Valério et al., 2016a).

5.2.4 Relative expression levels of protein phosphatase gene

For more than two decades it has been known that MCs are inhibitors of protein phosphatase, PP2A, but recently it was revealed to be a two-step mechanism, first the microcystin binds to the enzyme to inactivate it and then forms a covalent adducts (Valério et al., 2016b).

The relative expression of protein phosphatase PP2A, gene *Cdc55*, presented an overall tendency for being underexpresed when compared with the control, Table 5.1. There is a dose-response among the lowest concentrations (1 nM and 10 nM), but higher MC-LR concentrations translate into lower relative gene expression changes. The concentration at which the relative expression was higher was at 100 nM although lower than the control. However, Valério et al. (2016a), that performed the same RT-qPCR assay in *S.cerevisiae* exposed to 1 nM, 100 nM and 1 µM of MC-LR, presented different results. At the lower MC-LR concentration (1 nM) the gene was overexpressed, and observed a repression with 100 nM and 1 µM of MC-LR, when compared with the control condition (Valério et al., 2016a). This is in accordance with the explanation that genes overexpression may suggest that they are trying to overcome the effects of the toxin, when the toxin concentration is low, however, if the toxin concentration is higher it results in the repression of genes which may suggest that the DNA had extended deleterious effects that could not be overcome by the repair mechanism. To further support this explanation some studies using *Corbicula fluminea* (clam), showed that after exposure to MCs, PP2A gene expression levels increases replacing the enzyme deficiency, this might be the way that the organism tries to respond to the toxin, which inhibits PP2A (Valério et al. 2016b).

Table 5.1: *Summary of the relative gene expression changes when compared with the control condition. Overexpression (↗ arrow pointing above) or underexpression (↘ arrow pointing below). Equal expression (=) and similar expression (≈).*

	BER GENES					PP2A
MC-LR concentrations	***Apn1***	***Apn2***	***Rad27***	***Ntg1***	***Ntg2***	***Cdc55***
1 nM	↘	↘	≈	↘	↘	↘
10 nM	↘	↘	↗	↘	↘	↘
100 nM	↗	≈	↗	≈	↘	↘
1 µM	↗	=	↗	↘	↘	↘

Some of the results of this thesis regarding *Ntg1*, *Ntg2* and *Cdc55* genes are in fully accordance with Valério et al. (2016a), although the tendency of the exposure to different MC-LR concentrations is maintained, the controls conditions CT values presented very different values. This might be due to the fact that the assays performed in Valério et al. (2016a) were preliminary.

6. Conclusion

6.1 Conclusion and Final Considerations

As final conclusions of this work it can be affirmed that some of these assays should be repeated and reformulated, so that a clear final conclusion could be drawn about microcystin cytotoxic and genotoxic effects on the model organism *S. cerevisiae*, and complement the few reported information that currently exists.

The MTT assay could have given an idea about cell viability when exposed to different MC-LR concentrations. Two positive controls, SDS and H_2O_2 where used with success in the organism model *S. cerevisiae*. Apparently, MC-LR is not cytotoxic for *Saccharomyces cerevisiae*, although these results should be confirmed with other methods that accessed cell viability (e.g. flow cytometry or Neutral Red). Because of this no conclusion could be drawn about the maximum concentration to which the *S. cerevisiae* cells can be exposed without cells structure being compromised.

The genotoxic effects of MC-LR could not be assessed by the yeast comet assay because satisfactory and reproducible results were not obtained, despite all the attempts made. Thus, it could not be known which type of DNA damages: single breaks or double breaks, the toxin (MC-LR) may cause. If the comet tails had been obtained with YCA, the length or the fluorescence intensity of them could have provided information about the level and type of DNA damage. Larger comet tails would indicate a more relaxed and fragmented DNA, single DNA breaks, which is able to run faster on the agarose gel; on the contrary, smaller comet tails indicate a lower level of DNA breaks or the predominance of double DNA breaks, which would run slower on the agarose gel. Different MC-LR concentrations would be expected to give different comet tail lengths, but unfortunately this could not be confirmed. Since there are few studies using YCA, it was first necessary to verify if the assay was working using a positive control, H_2O_2, and only after obtaining a confirmation it would be possible to apply the assay to cells exposed to MC-LR. In this study, the two first steps of the protocol were optimized: (1) destruction of the cell wall and (2) of the membrane, however, no DNA migration could be obtained. More work is needed to optimize the YCA protocol, and afterwards it would be interesting to see the results using MC-LR as a toxic compound.

The assessment of genotoxic effects was also exploited using RT-qPCR assay, when evaluating relative gene expression of *Apn1*, *Apn2*, *Rad27*, *Ntg1*, *Ntg2 and Cdc55 S. cerevisiae* genes, after exposing the cells to different concentrations of MC-LR. These genes were chosen because they are involved in genotoxicity and in DNA repair systems. No statistical test could be applied to the results, because the reference genes *Alg9* and *Taf10* varied widely in some samples. Given this situation, we could not always obtain three biological replicas, and therefore no statistical test could be applied, and therefore currently there are only trends towards the control situation. Even though the results are preliminary, they seem interesting and are relatively in accordance with what was expected. Apparently, MC-LR affects both pathways of BER DNA repair system mechanism, and the gene *Cdc55* (coding for the protein phosphatase PP2A).

7. Bibliography and Sitegraphy

7.1 Bibliography

- Azevedo, F.; Marques, F.; Fokt, H.; Oliveira, R.; Johansson, B. Measuring oxidative DNA damage and DNA repair using the yeast comet assay. *Yeast*, **2011**, 28, 55–61.
- Azevedo, S.M.; Carmichael, W.W.; Jochimsen, E.M.; Rinehart, K.L.; Lau, S.; Shaw, G.R.; Eaglesham, G.K. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology*, **2002**, 181, 441—446.
- Azqueta, A.; Shaposhnikov, S.; Collins, A. R. DNA Repair Measured by the Comet Assay. InTech, **2011**, 615–636.
- Azqueta and Collins. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch Toxicol*, **2013**, 87, 849-867.
- Bernas, T.; Dobrucki, J.W. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. *Cytometry*, **2002**, 47, 236–42.
- Boiteux, S.; Jinks-Robertson, S. DNA repair mechanisms and the bypass of DNA damage in *Saccharomyces cerevisiae*. *Genetics*, **2013**, 193(4), 1025-64.
- Briand, J.F.; Jacquet, S.; Bernard, C.; Humbert, J.F. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. *Vet Res*., **2003**, 34, 361–377.
- Brinkman, D. L.; Bourne, D. G., **2013**. Handbook of Proteolytic Enzymes, third ed. Elsevier, 1726-1731.
- Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; Vandesompele, J.; Wittwer, C.T. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem*., **2009**, 55(4), 611−22.
- Campos, A.; Vasconcelos, V. Molecular Mechanisms of Microcystin Toxicity in Animal Cells. *Int. J. Mol. Sc*., **2010**, 11(1), 268−287.
- Carvalho, D.O.; Oliveira, R.; Johansson, B.; Guido, L.F. Dose-Dependent Protective and Inductive Effects of Xanthohumol on Oxidative DNA Damage in *Saccharomyces cerevisiae*. *Food Technology and Biotechnology*, **2016**, 54(1), 60–69.
- Chen, L.; Li, S.; Guo, X.; Xie, P.; Chen, J. The role of GSH in microcystin-induced apoptosis in rat liver: Involvement of oxidative stress and NF-kappaB. *Environ. Toxicol.*, **2014**, 31, 552−560.
- Chen, J.; Xie, P.; Li, L.; Xu, J. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. *Toxicol. Sci*., **2009**, 108, 81–89.
- Cho, U.S.; Xu,W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature*, **2007**, 445, 53−57.
- Chong, M.W.K.; Gu, K.D.; Lam, P.K.S.; Yang, M.; Fong, W.F. Study on the cytotoxicity of microcystin-LR on cultured cells. *Chemosphere*, **2000**, 41, 143−147.
- Corthell, J. T., **2014**. Reverse Transcription (RT) and Polymerase Chain Reaction (PCR). Basic Molecular Protocols in Neuroscience: Tips, Tricks, and Pitfalls. Pp. 27–40.
- Dheda, K.; Huggett, J.F.; Chang, J.S.; Kim, L.U.; Bustin, S.A.; Johnson, M.A.; Rook, G.A.; Zumla, A. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal. Biochem.*, **2005**, 344, 141–143.
- Dias, E.; Andrade, M.; Alverca, E.; Pereira, P.; Batoréu, M.C.C.; Jordan, P.; Silva, M.J. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from *Microcystis aeruginosa* on a kidney cell line. *Toxicon*, **2009**, 53, 487–495.
- Ding, W.X.; Nam Ong, C. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett*., **2003**, 220, 1−7.
- Eisenberg, E.; Levanon, E.Y. Human housekeeping genes are compact. *Trends in Genetics*, **2003**, 19(7), 362–365.
- Fischer, A.; Hoeger, S.J.; Stemmer, K.; Feurstein, D.J.; Knobeloch, D.; Nussler, A.; Dietrich, D.R. The role of organic anion transporting polypeptides (OATPs/SLCOs) in the toxicity of different

microcystin congeners in vitro: A comparison of primary human hepatocytes and OATP-transfected HEK293 cells. *Toxicology and Applied Pharmacology*, **2010**, 245, 9−20.

- Fraser, J.L.; Neill, E.; Davey, S. Fission yeast Uve1 and Apn2 function in distinct oxidative damage repair pathways in vivo. *DNA Repair (Amst)*, **2003**, 2(11), 1253−67.
- Fridman, J.S.; Lowe, S.W. Control of apoptosis by p53. *Oncogene*, **2003**, 22 (56), 9030−9040.
- Haber, J. E. Mating-Type Genes and MAT Switching in *Saccharomyces Cerevisiae*. Genetics, 2012, 191(1), 33−64.
- Hoeijmakers, J.H. Genome maintenance mechanisms for preventing cancer. *Nature*, **2001**, 411(6835), 366–37.
- Hudnell, H.K. The state of U.S. freshwater harmful algal blooms assessments, policy and legislation. Toxicon, **2010**, 55(5), 1024−1034.
- Kiruthika, B.; Padma, P.R. Zea mays leaf extracts protect *Saccharomyces cerevisiae* cell against oxidative stress-induced cell death. *Journal of Acute Medicine*, **2013**, 3, 83−92.
- Li, J.; Song, L. Applicability of the MTT assay for measuring viability of cyanobacteria and algae, specifically for *Microcystis aeruginosa* (Chroococcales, Cyanobacteria). *Phycologia*, **2007**, 46(5), 593–599.
- MacKintosh, C.; Beattie, K.A.; Klumpp, S.; Cohen, P.; Codd, G.A. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett*., **1990**, 264, 187−192.
- Maclean, M.J.; Aamodt, R.; Harris, N.; Alseth, I.; Seeberg, E.; Bjørås, M.; Piper, P.W. Base excision repair activities required for yeast to attain a full chronological life span. *Aging Cell*, **2003**, 2, 93 – 104.
- Marques, F.; Azevedo, F.; Johansson, B.; Oliveira, R. Stimulation of DNA repair in *Saccharomyces cerevisiae* by Ginkgo biloba leaf extract. *Food and Chemical Toxicology*, **2011**, 49, 1361–1366.
- Maynes, J.T.; Luu, H.A.; Cherney, M.M.; Andersen, R.J.; Williams, D.; Holmes, C.F.; James, M.N. Crystal structures of protein phosphatase-1 bound to motuporin and dihydromicrocystin-LA: elucidation of the mechanism of enzyme inhibition by cyanobacterial toxins. *J. Mol. Biol*., **2006**, 356, 111−120.
- Menacho-Márquez, M.; Murguía, J. Yeast on drugs: Saccharomyces cerevisiae as a tool for anticancer drug research. *Clinical and Translational Oncology*, **2007**, 9,221-228.
- Miloshev, G.; Mihaylov, I.; Anachkova, B. Application of the single cell gel electrophoresis on yeast cells. *Mutation Research*, **2002**, 69–74.
- Morris, L.P.; Degtyareva, N.; Sheppard, C.; Heyburn, L.; Ivanov, A.A.; Kow, Y.W.; Doetsch, P.W. Saccharomyces cerevisiae Apn1 Mutation Affecting Stable Protein Expression Mimics Catalytic Activity Impairment: Implications for Assessing DNA Repair Capacity in Humans. *DNA Repair (Amst)*, **2012**, 11(9), 753–765.
- Ohnishi, G.; Daigaku, Y.; Nagata, Y.; Ihara, M.; Yamamoto, K. *Saccharomyces cerevisiae* RAD27 complements its Escherichia coli homolog in damage repair but not mutation avoidance. *Genes Genet. Syst.*, **2004**, 79(3), 183-187.
- Oliveira, R.; Johansson, B. Quantitative DNA Damage and Repair Measurement with the Yeast Comet Assay. *Methods in Molecular Biology*, **2012**, 920, 101−109.
- Paerl, H.W.; Fulton, R.S.; Moisander, P.H.; Dyble, J. Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *Scientific World*, **2001**, 1, 76—113.
- Petrat, F.; Pindiur, S.; Kirsch, M., de Groot H. NAD(P)H, a primary target of 1O2 in mitochondria of intact cells. *J. Biol. Chem*., **2003,** 278, 3298–307.
- Peycheva, E.; Georgieva, M.; Miloshev, G. Comparison between alkaline and neutral variants of yeast comet assay. *Biotechnol. & Biotechnol*. *Eq*., **2009**, 23(1), 1090−1092.
- Pfaffl, M. W.; Vandesompele, J.; Kubista, M., **2009**. Data Analysis Software. Real-Time PCR: Current Technology and Applications, first ed. *Caister Academic Press*, pp. 65–83.
- Rank, J.; Syberg, K.; Jensen, K. Comet assay on tetraploid yeast cells. *Mutation Research*, **2009**, 673, 53–58.

- Ribeiro, G.F.; Côrte-Real, M.; Johansson, B. Characterization of DNA Damage in Yeast Apoptosis Induced by Hydrogen Peroxide, Acetic Acid, and Hyperosmotic Shock. *Mol. Biol. Cell.*, **2006**, 17(10), 4584–4591.
- Saravanan, B.C.; Sreekumar, C.; Bansal, G.C.; Ray, D.; Rao, J.R.; Mishra, A.K. A rapid MTT colorimetric assay to assess the proliferation index of two Indian strains of *Theileria annulata*. *Vet. Parasitol.*, **2003**, 113, 211–6.
- Sarmaa, M.K.; Kaushika, S.; Goswamib, P. Cyanobacteria: A metabolic power house for harvesting solar energy to produce bio-electricity and biofuels. *Biomass and Bioenergy*, **2016**, 90, 187–201.
- Staneva, D.; Peycheva, E.; Georgieva, M.; Efremov, T.; Miloshev, G. Application of comet assay for the assessment of DNA damage caused by chemical genotoxins in the dairy yeast *Kluyveromyces lactis*. *Antonie van Leeuwenhoek*, **2013**, 103, 143–152.
- Stellwagen; Stellwagen. The free solution mobility of DNA in Tris-acetate-EDTA buffers of different concentrations, with and without added NaCl. *Electrophoresis*, **2002**, 23(12), 1935–1941.
- Stephenson, F.H., **2010**. Calculations for Molecular Biology and Biotechnology. A Guide to Mathematics in the Laboratory, second ed. Elsevier.
- Stockert, J.C.; Blázquez-Castro, A.; Cañete, M.; Villanueva, A. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta histochemica*, **2012**, 114(8), 785−96.
- Sun, Y.; Zheng, Q.; Sun, Y.T.; Huang, P.; Guo, Z.L.; Xu, L.H. Microcystin-LR induces protein phosphatase 2A alteration in a human liver cell line. *Environ.Toxicol*., **2014**, 29, 1236−1244.
- Teste, M.; Duquenne, M.; François, J.M.; Parrou, J. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. *BMC Mol. Biol.*, **2009**, 10, 99.
- Teparic, R.; Stuparevic, I.; Mrsa, V. Increased mortality of *Saccharomyces cerevisiae* cell wall protein mutants. *Microbiology*, **2014**, 150, 3145-3150.
- Thomas, P.; Sekhar, A.C.; Upreti, R.; Mujawar, M.M.; Pasha, S.S. Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples. *Biotechnology Reports*, **2015**, 8, 45–55.
- Valério, E.; Campos, A.; Osório, H.; Vaconcenlos, V. Proteomic and Real-Time PCR analyses of Saccharomyces cerevisiae VL3 exposed to microcystin-LR reveals a set of protein alterations transversal to several eukaryotic models. *Toxicon*, **2016a**, 112, 22−28.
- Valério, E.; Vasconcelos, V.; Campos, A. New Insights on the Mode of Action of Microcystins in Animal Cells - A Review. *Mini-Reviews in Medicinal Chemistry*, **2016b**, 16, 000−000.
- Valério, E.; Vilares, A.; Campos, A.; Pereira, P., Vasconcelos, V. Effects of microcystin-LR on *Saccharomyces cerevisiae* growth, oxidative stress and apoptosis. *Toxicon*, **2014**, 90, 191−198.
- Van der Merwe, D., **2014**. Freshwater cyanotoxins. Biomarkers in Toxicology, pp. 539-548.
- Van Meerloo, J.; Kaspers, G.J.; Cloos, J. Cell sensitivity assays: the MTT assay. Methods *Mol. Biol.*, **2011**, 731, 237–45.
- Vincent, W.F., **2009**. Encyclopedia of Inland Waters. *Gene Likens*, pp. 226-232.
- Von Ahsen, N.; Oellerich, M.; Armstrong, V.W.; Schütz, E. Application of a thermodynamic nearest-neighbor model to estimate nucleic acid stability and optimize probe design: prediction of melting points of multiple mutations of apolipoprotein B-3500 and factor V with a hybridization probe genotyping assay on the LightCycler. *Clinical Chemistry*, **1999**, 45, 2094-2101.
- Wang, X.; Chen, Y.; Zuo, X.; Ding, N.; Zeng, H.; Zou, X.; Han, X. Microcystin (-LR) induced testicular cell apoptosis via up-regulating apoptosis-related genes in vivo. *Food Chem. Toxicol.*, **2013**, 60, 309−317.
- Warringer, J.; Zörgö, E.; Cubillos, F.A.; Zia, A.; Gjuvsland, A.; Simpson, J.T.; Forsmark, A.; Durbin, R.; Omholt, S.W.; Louis E.J.; Liti, G.; Moses, A.; Blomberg, A. Trait variation in yeast is defined by population history. *PLoS Genet*., **2011**, 7(6), e1002111.
- Zhou, M.; Tu, W.; Xu, J. Mechanisms of microcystin-LR-induced cytoskeletal disruption in animal cells. *Toxicon*, **2015**, 101, 92−100.

7.2 Sitegraphy

- CDC55. (2017). http://www.yeastgenome.org/locus/S000003158/overview. Acessed on January 2nd of 2017.
- Comet Assay. (2017). http://cometassay.com/index_files/Page345.htm. Accessed on March 12th of 2017.
- Molecular Devices. (2017). https://www.moleculardevices.com/applications/areas-research/cytotoxicity. Accessed on February 10th of 2017.
- MTT Cell Proliferation Assay. (2017). https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx. Accessed on January 9th of 2017.
- OD660 vs Number of Cells. (2016). http://www.pangloss.com/seidel/Protocols/ODvsCells.html. Accessed on November 11th of 2016.
- Oxford Gene Technology. (2017). http://www.ogt.co.uk/resources/literature/483_understanding_and_measuring_variations_in_dna_sample_quality. Accessed on June 24th 2016.
- Saccharomyces Genome Database. (2016). http://www.yeastgenome.org/. Accessed on May 3rd 2016.
- Sigma Aldrich. (2017). http://www.sigmaaldrich.com/catalog/product/sigma/l5125?lang=pt&region=PT. Accessed on March 12th of 2017.
- Simgene. (2016). http://www.simgene.com/. Accessed on February 25th of 2017.
- World Health Organization. (2016). http://www.who.int/water_sanitation_health/dwq/chemicals/microcystin.pdf. Accessed on December 17th of 2016.

8. Annexes

ANNEX I

Culture medium composition

Table 8.1: *YPD liquid medium composition used for S. cerevisiae growth. The components amounts were added to 500 mL of deionized water*

Stock solution	Components	Components (g)
YPD (500 mL)	Yeast extract (1%)	5
	Peptone (2%)	10
	Glucose (2%)	10

YEPD liquid medium also known as YPD liquid medium, was prepared using yeast extract (Oxoid®), peptone (Difco®) and glucose (Merck®) in a 500 mL Schott flask. After the components have solubilized, the medium was sterilized in the autoclave for 20 min at 120ºC.

Table 8.2: *YPD solid medium composition used in S. cerevisiae growth. The components amounts were added to 500 mL of deionized water*

Stock solution	Components	Components (g)
YPD (500 mL)	Yeast extract (1%)	5
	Peptone (2%)	10
	Glucose (2%)	10
	Agar (2%)	10

YPD solid medium was prepared the same way as the liquid YPD with the addition of agar (Oxoid®). With the YPD medium still warm, we pour about 20 mL in aseptic 90 mm Petri dishes, let it cool down and then stored them in the refrigerator at 4ºC for posterior use.

ANNEX II

MTT assay

Material and Equipment

- Eppendorfs 1.5 mL
- Microcystin-LR stock solutions of 1 mg/mL; 100 µg/mL; 10 µg/mL; 1 µg/mL
- Fitoclima (Hera s600 Aralab®)
- 2 Spectrophotometer (ThermoFisher Labsystems®); (Sherwood®)
- Gloves
- Micropipettes and sterilized tips
- 6 well plate (Starstedt®)
- MTT (Sigma®)
- DMSO (ThermoFisher®)
- 96 well plate (Starstedt®)

Procedure

Day 1

1. *Saccharomyces cerevisiae* cells were grown in standard YPD medium overnight in a FitoClima at 20ºC

Day 2

2. Inoculate 2 mL YPD medium in a 6 well plate, with a cell density adjusted to O.D 0.05, corresponding approximately to 5 x 10^5 cells/mL
3. The plate with the following treatments was placed 4 hours in the fitoclima at 20ºC:

   1) YPD + pre-inoculum (control)
   2) YPD + pre-inoculum + 2 µL de MC-LR 1 mg/mL (1 µM final concentration)
   3) YPD + pre-inoculum + 2 µL de MC-LR 100 µg/mL (100 nM final conc.)
   4) YPD + pre-inoculum + 2 µL de MC-LR 10 µg/mL (10 nM final conc.)
   5) YPD + pre-inoculum + 2 µL de MC-LR 1 µg/mL (1 nM final conc.)

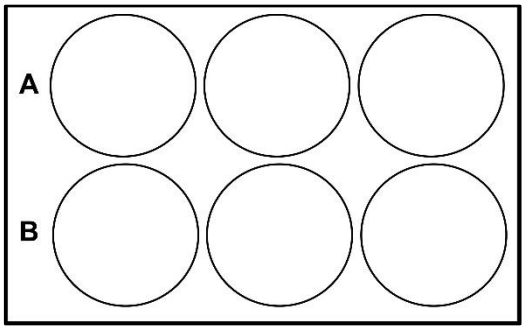


Figure 8.1: *Scheme of the 6 well plate (http://www.cellsignet.com/media/templ.html)*

4. 1 mL of the suspension from each well was harvest to a 1.5 mL eppendorf
5. The suspension was centrifuged for 5 min at 8.000 g
6. The cells were resuspended in 100 µL of PBS
7. Then 100 µL of MTT [3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide] solution 0.5 mg/mL was added [1]
8. The suspension was incubated in the fitoclima at 20ºC in an agitator for 2/3 hours in the dark

[1] Dissolve the MTT in PBS at 0.5mg/mL final concentration

9. Centrifuge the suspension for 5 minutes at 8.000 g
10. The cells were resuspended in 300 µL DMSO
11. Inoculate 100 µL of each treatment in a 96 well plate (3 replicates)
12. Skate the 96 well plate for at least 15 minutes in the dark

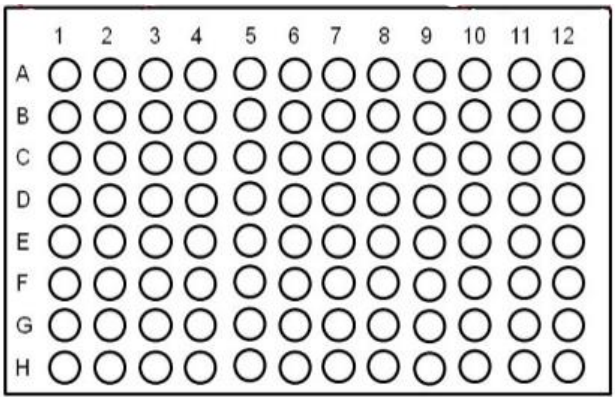


Figure 8.2: *Scheme of a 96 well plate (http://www.cellsignet.com/media/templ.html)*

13. Measure the absorbance of the wells, including the blanks, at 570nm with a reference wavelength of 690 nm. The blanks should give values of 0.1 O.D. units

References:

Teparic, R.; Stuparevic, I.; Mrsa, V. Increased mortality of *Saccharomyces cerevisiae* cell wall protein mutants. *Microbiology*, **2014**, 150, 3145-3150.

NOTES: Development of dark color or formation of crystals indicate product deterioration.

ANNEX III

Comet assay

Material and Equipment

- Eppendorfs 1.5 mL
- Different concentrations of Hydroxide peroxide (H_2O_2) and Microcystin-LR stock solutions at 1 mg/mL; 100 µg/mL; 10 µg/mL; 1 µg/mL
- Fitoclima (Hera®)
- Spectrophotometer (Sherwood®)
- Gloves
- Micropipettes and sterilized tips
- 6 well plate (Starstedt®)
- Freezing plate
- Thermoblock
- Low melting agarose 1.5%
- Glass slides coated with 1% agarose
- Lyticase (Sigma®)
- Electrophoresis buffer
- Lysis buffer
- Neutralization buffer
- PBS
- Fluorescence microscope
- GelRed

Procedure

DAY 1

1. *Saccharomyces cerevisiae* cells were grown in standard YPD medium overnight in a Fitoclima at 20ºC

DAY 2

2. Set the thermoblock to 90ºC and place the eppendorf with low melting agarose 1.5% for about 10 minutes, until it melts
3. Set the thermoblock to 35ºC and keep the eppendorf with the agarose there to achieve the optimum temperature and be used later, minimum time is 35 minutes, so it doesn't destroy the cells when used
4. Adjust cell density to an O.D 0.05, corresponding approximately to 5,5 x 10^5 cells/mL
5. Inoculate 5 mL in a six wells plate
6. Each well was exposed to determinate treatment such as different concentrations of hydroxide peroxide (positive controls) or different MC-LR concentrations
7. The plate with the following treatments was exposed 4 hours in the FitoClima at 20ºC ± 2ºC in a shaker
8. 1 mL of the suspension of every well was harvest to a 2 mL eppendorf
9. The suspension was centrifuged for 2 minutes at 2.000 g
10. Discard the supernatant and keep the eppendorf with the pellet in ice(1)
11. Wash the cells with 1mL of ice cold deionised water twice
12. The suspension was centrifuged for 2 minutes at 2.000 g

13. Resuspend the pellet in 40 µL of lyticase 2 mg/mL (which was previously diluted in S-Buffer)
14. Incubate 30 minutes on the ThermoBlock, 500 rpm at 35°C.
15. Centrifuge for 2 minutes at 2.000 g and discard the supernatant
16. Wash the cells with 100 µL of S-buffer
17. Centrifuge for 2 minutes at 2.000 g and discard the supernatant
18. [(2)] Resuspend with 1mL of 10 mM H_2O_2 and incubate for 20 minutes at 4ºC
19. [(2)] Centrifuge for 2 minutes at 2.000 g and discard the supernatant
20. Resuspend the cells with S-buffer, to obtain 60 µL of pellet
21. Add 100 µL of low melting agarose 1.5% to the sample and mix it well
22. Dispose 40 µL of each solution in a glass slide previously coated with agarose 1%, each 40 µL will correspond to a gel and usually each slide caries two gels
23. Place a coverslip on each gel and immediately place the glass slides on top of a cold plate
24. Place all the glass slides in a box previously covered with aluminum foil
25. Incubate the slides at 4ºC for 2 minutes
26. Remove the coverslips of each gel and incubate the slides for 30 minutes in lyses solution (4ºC), to lyse the spheroplasts
27. Wash the slides with neutralization buffer (4ºC) for 5 minutes, repeat this step twice
28. Pour about 1300 mL of electrophoresis buffer (4ºC, pH 10) on an electrophoresis tank
29. Transfer the slides to electrophoresis buffer (4ºC) for 20 minutes, to unwind the DNA
30. Set the chosen volts charge and submit the samples to electrophoresis 0.7 V/cm for 10 minutes
31. Place the slides in neutralization buffer (4ºC) for 10 minutes
32. Keep the slides at room temperature for 2 to 3 days in an aluminum foil covered box, so that the samples are in the dark (dehydrate the agarose)

Day 3

33. Stain the slides with 36 µL of GelRed for each gel with a concentration of 1:10000 (1µL to 10mL of water)
34. Place slides over the gels and lay the glass slides on a covered box containing wet cotton in the dark at the refrigerator (so that the gels do not dry and the cells dehydrate)
35. Obtain 50 images for each concentration at a magnification of 100x in a fluorescence microscope
36. The images were analyzed with the aid of the program / software CometScoreTM and analytical parameter Tail Length (mM) was chosen to determine the DNA damage. In each slide were analyzed at least 50 comets and the error bars represent variabilities between at least 3 different blades obtained in 3 biological replicas.

[(1)] From this step forward work with iced solutions (4ºC) and keep the pellet refrigerated

[(2)]These protocol steps were only performed in some comet assays (5,6 and 7)

Comet Solutions:

Table 8.3: *Lysis buffer 1 composition. The components amount were added to 250 mL of deionized water*

Stock solution	Components (mM)	pH
Lysis buffer (1)	NaOH (30 mM)	12
	NaCl (1 M)	
	Tris-HCl (10 mM)	
	EDTA (50 mM)	
	N-laurylsarcosine (0.1% w/v)	

Table 8.4: *Lysis buffer 2 composition. The components amount were added to 250 mL of deionized water*

Stock solution	Components (mM)	pH
Lysis buffer (2)	NaOH (30 mM)	12
	NaCl (1 M)	
	Tris-HCl (10 mM)	
	EDTA (50 mM)	
	Triton-X-100 (1% v/v)	

Lysis buffer was prepared the day before it is used in a 250 mL flask, the volume was completed with sterile water. After the components have solubilized, the pH of the solution is adjusted to 12.

Table 8.5: *Electrophoresis buffer 1 composition. The components amount were added to 2 L of deionized water*

Stock solution	Components (mM)	pH
Electrophoresis buffer (1)	NaOH (30 mM)	10
	Tris-HCl (10 mM)	
	EDTA (10 mM)	

Table 8.6: *Electrophoresis buffer 2 composition. The components amount were added to 2 L of deionized water*

Stock solution	Components (mM)	pH
Electrophoresis buffer (2)	NaOH (300 mM)	10
	NaCl (1 M)	
	N-laurylsarcosine (0.05% w/v)	
	Tris-HCl (10 mM)	
	EDTA (1 mM)	

Table 8.7: *Electrophoresis buffer 3 composition. The components amount were added to 2 L of deionized water*

Stock solution	Components (mM)	pH
Electrophoresis buffer (3)	NaOH (300 mM)	13
	NaCl (1 M)	
	N-laurylsarcosine (0.05% w/v)	
	EDTA (1 mM)	

Electrophoresis buffer does not need to be prepared in the same day or the day before the comet assay is made. The solution is prepared in a 2000 mL flask, the volume was completed with sterile water. After the components have solubilized, the pH of the solution is adjusted to 13 with hydrogen chloride (HCl) and sodium hydroxide (NaOH).

Table 8.8: *Neutralization buffer composition. The components amount were added to 250 mL of deionized water*

Stock solution	Components (mM)	pH
Neutralization buffer	Tris-HCL (10 mM)	7.4

Neutralization buffer does not need to be prepared in the same day or the day before the comet assay is made. The solution is prepared in a 250 mL flask, the volume was completed with sterile water.

Table 8.9: *S-buffer composition. The components amount were added to 250 mL of deionized water*

Stock solution	Components (mM)	pH
S-buffer	Sorbitol (1 M)	6.5
	Monopotassium phosphate (25 mM)	

S-buffer does not need to be prepared in the same day or the day before the comet assay is made. The solution is prepared in a 250 mL flask, the volume was completed with sterile water and kept in the fridge until it is used.

References:

Oliveira, R.; Johansson, B. Quantitative DNA Damage and Repair Measurement with the Yeast Comet Assay. *Methods in Molecular Biology*, **2012**, 920, 101−109.

ANNEX IV

DNA extraction protocol

(DNA extraction kit: Spin Plant Mini Kit (Invitek®))

Material and Equipment

- Kit materials and solutions
- Centrifuge (Eppendorf 5415C Centrifuge®)
- Water bath at 65ºC
- Vortex (Heidolph®)
- Gloves
- Micropipettes and tips

Procedure

1. Centrifuge 1 mL of pre-inoculum, prepared previously until you have about 60 mg of pellet on a 1.5 mL reaction tube. Discard supernatant.
2. Add 400 µL Lysis Buffer P and 20µL Proteinase K and homogenize on the vortex
3. Incubate at 65ºC for 30 minutes
4. Transfer the solution onto the pre-filter
5. Centrifuge for 1 minute at 13.400 x *g*
6. Discard the pre-filter
7. Add 200 µL of Binding Buffer P and vortex it
8. Transfer the suspension onto the spin filter
9. Incubate for 1 minute
10. Centrifuge at 13.400 g for 1 minute
11. Discard the filtrate
12. Add 550 µL of Wash Buffer I
13. Centrifuge at 13.400 x g for 1 minute
14. Discard the filtrate
15. Place the spin filter again into the 2 mL receiver tube
16. Add 550 µL of Wash Buffer II
17. Centrifuge at 13.400 *x g* for 1 minute
18. Discard the filtrate
19. Repeat the washing step once again
20. Discard the filtrate and centrifuge at 13.400 *x g* for 2 minutes
21. Place the spin filter into a new 1.5 mL receiver tube and add 100 µL of the pre-warmed Elution Buffer D
22. Incubate for 3 minutes
23. Centrifuge at 9.*300 x g* for 1 minute
24. Discard the spin filter
25. Close the receive tubes and store the DNA samples at 4ºC or -20ºC

ANNEX V

RNA extraction protocol

Material and Equipment

- Ice
- Isopropanol
- Microcentrifuge at 4ºC (Eppendorf®)
- Ethanol 75%
- Vortex (Heidolph®)
- Glass beads 425-600 µm (Sigma)
- Gloves
- Chloroform
- Micropipettes and tips
- TRIzol® reagent (Invitrogen)
- Sterilized water

Procedure

1. Place ice in a container
2. Set the microcentrifuge at 4ºC
3. Set a water bath at 30ºC
4. Add about 100 µL glass beads to the 2 mL eppendorfs that contain the cell pellets after defrosting them (eppendorf with the pellet were kept at -80ºC in the ultralow temperature freezer)
5. Add 1 mL of TRIzol® reagent (invitrogen®) to the eppendorfs
6. Expose the cells to 6 cycles of 1 minute in the vortex and 30 seconds on ice
7. Place the eppendorfs in the 30ºC water bath for 5 minutes
8. Add 0.2 mL of chloroform
9. Stir vigorously for 15 seconds
10. Place the eppendorfs at the 30ºC water bath for 3 minutes
11. Centrifuge for 15 minutes at 12000 *x g*, 4ºC
12. Transfer the aqueous phase (transparent upper part) into a sterile and cold 1.5 mL eppendorf
13. Add 0.6 mL of isopropanol
14. Place the eppendorfs at the 30ºC water bath for 10 minutes
15. Centrifuge for 10 minutes at 12000 *x g*, 4ºC
16. Discard the supernatant by inversions and then remove the remaining supernatant with a pipette
17. Wash the pellet with 1 mL of ice-cold ethanol 75%
18. Centrifuge for 5 minutes at 7500 *x g*, 4ºC
19. Remove the ethanol by inversion and let the pellet dry at room temperature
20. Dissolve the pellet with 40 µL of sterilized water (RNase free) (Gibco®) and quantify the concentration by spectrophotometry (NanoDrop®)
21. Store at - 80ºC in the ultralow temperature freezer if the RNA is not used immediately

References:

Valério, E.; Campos, A.; Osório, H.; Vaconcenlos, V. Proteomic and Real-Time PCR analyses of *Saccharomyces cerevisiae* VL3 exposed to microcystin-LR reveals a set of protein alterations transversal to several eukaryotic models. *Toxicon*, **2016a**, 112, 22−28.

ANNEX VI

RNA purification protocol

(High Pure RNA Isolation Kit (Roche®))

Material and Equipment

- Kit materials and solutions
- PBS (Gibco®)
- Vortex (Heidolph®)
- Gloves
- RNA micropipettes and filter tips
- Sterilized water

Procedure

1. Add 90 µL of DNase Incubation Buffer at the 10 µL aliquot of DNase I, prepared previously. Mix it with a pipette
2. Add the mix directly above the membrane center of the filter tubes and let it stand at room temperature for 15 minutes
3. Add 500 µL of Wash Buffer I to the filter column and centrifuge at 8000 *x g* for 15 seconds. Discard the liquid at the column
4. Add 500 µL of Wash Buffer II to the filter column and centrifuge at 8000 *x g* for 15 seconds. Discard the liquid at the column
5. Add 200 µL of Wash Buffer II to the filter column and close the lid
6. Centrifuge for 2 minutes at 8000 *x g* so that the filter membrane dries
7. Transfer the column to a new tube of 1.5 mL
8. Add 50 µL of sterilized water (Gibco®) to the centre of the filter tubes membrane and let it set for 1 minute at room temperature
9. Close the tube and centrifuge for 1 minute at 8000 *x g* for RNA elution
10. Transfer the eluted solution at the eppendorf to the filter tubes membrane center and centrifuge 1 minute at 6.500 x g to elude the remaining RNA
11. Store at -80ºC in the ultralow temperature freezer if the RNA is not to be used immediately

After RNA purification, we need to perform a PCR with a reference gene (e.g. *Taf10*) to confirm if the RNA is not contaminated with DNA. There can be no product amplification.

ANNEX VII

Conventional PCR protocol

Material and Equipment

- Ice
- PCR tubes
- Kit solutions (Invitrogen®)
- DNA ladder (Invitrogen®)
- Loading buffer
- Vortex (Heidolph®)
- Gloves
- RNA micropipettes and filter tips
- Sterilized water
- Agarose

Procedure

1. Place ice in a container
2. Mark PCR tubes and place them in ice
3. Make a Mix, where the reagents quantities will depend on the number of samples tested. For 8 samples add 120.4 µL of RNase and DNase free water (Gibco®); 20.0 µL of 1x PCR Rxn buffer (Invitrogen®); 8.0 µL of dNTPs (Invitrogen®); 10.0 µL of each primer forward and reverse at 50 µM (Thermo Fisher Scientific®); 10.0 µL of 1% (v/v) W-1(Invitrogen®); 12.0 µL of $MgCl_2$ (Invitrogen®) and 1.6 µL of Taq DNA Polimerase(Invitrogen®)
4. Vortex the mix
5. Distribute 24 µL of the mix by the 8 PCR tubes
6. Place 1 µL of each RNA/DNA (100-200 ng) on the respective PCR tube; 1 µL of water on the PCR tube corresponding to the negative control and 1 µL of DNA to the PCR tube of the positive control
7. Place the PCR tubes on the thermocycler TGradient (Biometra®) and set the following parameters: Denaturation at 94ºC for 5 minutes; 35 cycles of the following settings: denaturation at 94ºC for 45 seconds, anneling at 50ºC for 45 seconds and extension at 72ºC for 45 seconds; final step at 72ºC for 5 minutes
8. Assemble the electrophoresis equipment
9. Measure 0.4 g of agarose (1%) and add 40 mL of Tris-EDTA buffer (TE) 0.5x
10. Dissolve it on the microwave and add 1µL of GelRed Nucleotic Acid Strain (Biotium®) while the solution is still hot
11. Pour the agarose on the electrophoresis tray, place the comb, and let it solidify
12. Remove the combs and the lateral rubbers from the tray and place it onto the electrophoresis buffer chamber, filled with TE buffer 0.5x
13. In the first well pour 5 µL of 1 Kb Plus DNA Ladder
14. For each sample mix 3 µL of the sample with 3 µL of loading buffer (LB)
15. Place each sample into a well
16. Close the electrophoresis buffer chamber and turn on the power at 80 volts for 45 minutes
17. Turn off the equipment and visualize the gel to a UV PCR chamber.

ANNEX VIII

Primers design

Procedure

1. Download *Saccharomices cerevisiae* VL3 genes, genomic DNA, of interest from the site "www.yeastgenome.com" and save it on the computer
2. Upload the sequence on the site Simgene (from http://www.simgene.com/, accessed on February 2016), Primer3 tool
3. Select the primers specifications (e.g. the length of the primer (usually between 18 and 25 bp), the melting temperature (ideally between 53ºC and 63ºC) and the length of the product (that had to be between 100 and 300 bp for RT-qPCR assays))
4. Chose the primers that are closest to 150 bp in lenght and those who had at least 50% ratio between Citocines and Guanines, since they are more stable. Take into account that primers at the beginning or at the end of the sequence are not favorable for PCR amplification
5. For each primer analysis the probability of generating hairpins, heterodimers and selfdimers, was verified with OligoAnalyzer 3.1 software. Take into account that higher Gibbs free energy leads to a decreased in dimers formation since the process is not spontaneous, which in turn leads to a more stable primer.
6. Make a BLAST (Basic Local Alignment Search Tool) search. That allows the comparison of the selected primer sequence with database sequences in order to identify if there are others sequences that resemble the one selected, thus preventing non-specific amplifications